



Molecular systematics of flyingfishes (Teleostei: Exocoetidae): evolution in the epipelagic zone

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The flyingfish family Exocoetidae is a diverse group of marine fishes that are widespread and abundant in tropical and subtropical seas. Flyingfishes are epipelagic specialists that are easily distinguished by their enlarged fins, which are used for gliding leaps over the surface of the water. Although phylogenetic hypotheses have been proposed for flyingfish genera based on morphology, no comprehensive molecular studies have been performed. In the present study, we describe a species-level molecular phylogeny for the family Exocoetidae, based on data from the mitochondrial cytochrome *b* gene (1137 bp) and the nuclear RAG2 gene (882 bp). We find strong support for previous morphology-based phylogenetic hypotheses, as well as the monophyly of most currently accepted flyingfish genera. However, the most diverse genus *Cheilopogon* is not monophyletic. Using our novel flyingfish topology, we examine previously proposed hypotheses for the origin and evolution of gliding. The results support the progressive transition from two-wing to four-wing gliding. We also use phylogenetic approaches to test the macroecological effects of two life history characters (e.g. egg buoyancy and habitat) on species range size in flyingfishes. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 161–174.

ADDITIONAL KEYWORDS: Beloniformes – cytb – gliding strategies – mtDNA – nuclear DNA – phylogeny – RAG2.

INTRODUCTION

The marine epipelagic zone is one of the largest and most productive habitats on Earth, although it exhibits remarkably low species diversity (Angel, 1993). Survival in epipelagic habitats presents a number of specific challenges for fishes, including the rarity of substrate for egg deposition and refuges, and a highly patchy distribution of resources (Parin, 1968; Hamner, 1995; Allen & Cross, 2006). Epipelagic fishes are also exposed to predators and powerful abiotic forces (e.g. ocean currents) during all phases of their lifecycles. As a result, epipelagic species exhibit an

array of specialized adaptations. Reproductive characteristics that compensate for the absence of benthic substrate (e.g. buoyant eggs or egg filaments for attachment to floating debris and vegetation) and adaptations for predator avoidance (e.g. defensive spines, cryptic coloration, and protective schooling behaviour) are present in many epipelagic taxa (Hamner, 1995; Nelson, 2006). Among the most spectacular adaptations to epipelagic habitats is the aerial behaviour of flyingfishes (and certain species of squids), which make gliding leaps from the water, presumably to evade predators (Mohr, 1954; Evans & Sharma, 1963; Fish, 1990; Gillett & Ianelli, 1991; Davenport, 1992; Davenport, 1994; Kutschera, 2005).

The flyingfish family Exocoetidae includes approximately 50 species that are distributed across the tropical and subtropical regions of the Pacific,

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Atlantic, and Indian oceans. A key element of epipelagic food webs, flyingfishes feed on zooplankton and transfer energy from lower levels of the trophic system to top predators (Parin, 1968). As the predominant form of middle-sized nekton (i.e. actively swimming organisms < 1 m in length) in the open ocean, flyingfishes are a critical source of food for pelagic predators such as dolphinfishes, tunas, billfishes, cetaceans, and pelagic seabirds (Parin, 1968). The most distinct feature of flyingfishes is their greatly enlarged paired fins, which allow glides above the surface of the water (Davenport, 1994). Some species have greatly enlarged pectoral fins (two-wing gliders; also described as monoplane gliders by Breder, 1930), whereas others have greatly enlarged pectoral and pelvic fins (four-wing gliders; also described as biplane gliders by Breder, 1930). Four-wing flyingfishes can glide up to 400 m, and can accomplish turns and altitude changes, whereas two-wing gliders travel shorter distances, usually in a straight line (Davenport, 1994).

Flyingfishes show variation in life history and reproductive biology. Although all species are oviparous, some have specialized egg structures that allow attachment of eggs to floating debris and seaweed, whereas others lay buoyant eggs on the surface of the open ocean (Collette *et al.*, 1984). However, some species return to, or continuously occupy, coastal habitats to complete their life cycle, whereas others spend their entire lives far offshore in pelagic habitats. Geographical range size varies considerably among species, from locally restricted to circumtropical. For example, *Fodiator rostratus* is endemic to the nearshore waters of the eastern tropical Pacific (Parin, 1995), whereas *Exocoetus volitans* has a largely pantropical distribution (Parin & Shakhovskoy, 2000). Life-history traits, such as dispersal ability of eggs and larvae, have been demonstrated to affect geographic ranges of other marine taxa, including invertebrates and fishes (Bowen & Avise, 1990; Palumbi, 1992; Knowlton, 1993; Burton, 1998; Palumbi, 2004; Lester *et al.*, 2007; Galarza *et al.*, 2008; Eble, Toonen & Bowen, 2009; for a review, see Cowen & Sponaugle 2009). However, correlations between life history characters and species range size have not been investigated in flyingfishes.

The Exocoetidae has been proposed as a monophyletic group within the order Beloniformes based on both morphological (Bruun, 1935; Parin, 1961; Collette *et al.*, 1984; Dasilao & Sasaki, 1998) and molecular studies (Lovejoy, 2000; Lovejoy, Iranpour & Collette, 2004). However, phylogenetic hypotheses within the family Exocoetidae have been entirely based on morphological characters and have focused on genus-level relationships. Parin (1961; see also Bruun, 1935) proposed an evolutionary scheme

that grouped seven genera into four subfamilies: Fodiatorinae, Parexocoetinae, Exocoetinae, Cypselurinae (with the latter containing the genera *Prognichthys*, *Cypselurus*, *Cheilopogon*, and *Hirundichthys*) (Fig. 1A). Collette *et al.* (1984) produced a subfamily-level analysis, based on morphological characters, that matched Parin (1961) (Fig. 1A). More recently, Dasilao & Sasaki (1998; see also Dasilao, Sasaki & Okamura, 1997) produced a cladistic analysis based on 41 morphological characters (Fig. 1B), which provided further support for the trees proposed by Parin (1961) and Collette *et al.* (1984). The morphology-based trees suggest a stepwise evolution of gliding capability, progressing from two-wing gliding (*Fodiator*, *Parexocoetus*, and *Exocoetus*), to four-wing gliding (Cypselurinae). These authors also proposed that *Oxyporhamphus*, a taxon that shares features with both flyingfishes and halfbeaks, should be considered a basal member of Exocoetidae (Dasilao *et al.*, 1997). Lovejoy *et al.* (2004) presented a molecular phylogenetic analysis for beloniform fishes that included eight flyingfish species in seven genera. This analysis closely agreed with the flyingfish relationships based on morphological studies, although *Parexocoetus* and *Fodiator* were grouped as sister taxa.

Flyingfishes are an excellent group for studying the evolution of epipelagic adaptations, biogeography, and marine diversification. However, a species-level phylogeny is a prerequisite for such investigations. In the present study, we describe the first molecular

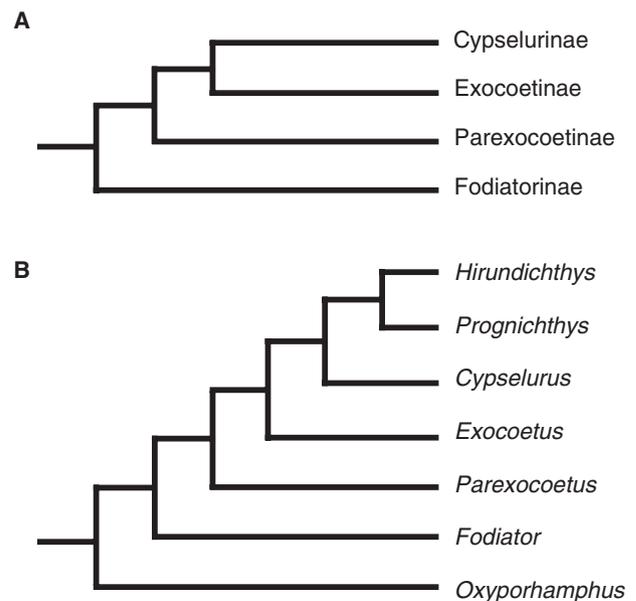


Figure 1. Phylogenetic hypotheses proposed for flyingfishes based on morphological characters. A, subfamily level tree proposed by Parin (1961), Collette *et al.* (1984). B, genus level tree proposed by Dasilao & Sasaki (1998).

phylogeny for the Exocoetidae based on mitochondrial and nuclear genes. Our objectives were: (1) to generate a species-level molecular phylogeny for flyingfishes and compare this with previous morphology-based hypotheses; (2) to test the monophyly of currently accepted flyingfish subfamilies and genera; (3) to reconstruct the evolution of flyingfish gliding strategies; and (4) to test whether species range size is correlated with variation in egg buoyancy and habitat preference.

MATERIAL AND METHODS

TAXON SAMPLING

Specimens were collected in the field or donated by collaborators, with tissues stored in 95% ethanol. Voucher specimens have been deposited in museum collections (Table 1). In total, 65 flyingfish individuals (representing 31 species and seven genera) and ten outgroup individuals (representing five species and four genera) were included.

MOLECULAR DATA COLLECTION

Genomic DNA was extracted using DNeasy kits (Qiagen). The mitochondrial cytochrome *b* (*cytb*) gene (1137 bp) was amplified by polymerase chain reaction (PCR) and primers ExoCBFwd (5'-GGACTTATGAYTTGAAAAACCATCGTTG-3') and ExoCBRev (5'-AACCTTCGACGTTTCGGCTTACAAGGCCG-3'), which were designed using published data from actinopterygian (Sevilla *et al.*, 2007) and beloniform fishes (Lovejoy, 2000). A portion of the recombination activating gene 2 (*RAG2*) (882 bp) was amplified using primers Ffly-Ch (5'-ACTGAGA TGAAGTTGAGACCCAT-3') and Rfly-Ch (5'-CCTCA GACTGGAAGCTCACCTG-3'), which were designed using published data from Beloniformes (Lovejoy & Collette, 2001; Lovejoy *et al.*, 2004).

PCR for *cytb* amplifications were performed with 8 mg/L of bovine serum albumin, 1 × Taq Polymerase Buffer, 0.2 mM of each dNTP, 2.8 mM MgCl₂, 7.5 mol of each primer, 1.25 U of Taq DNA Polymerase (Fermentas Inc.) and approximately 125 ng of genomic DNA. *RAG2* amplifications were performed with 8 mg/L of bovine serum albumin, 1 × Taq Polymerase Buffer, 0.2 mM of each dNTP, 2.0 mM MgCl₂, 5 mol of each primer, 1 U of Taq DNA Polymerase, and approximately 250 ng of genomic DNA.

PCR was performed using the following conditions for *cytb*: initial denaturation at 95 °C for 30 s; followed by 35 cycles of 95 °C for 30 s, 50 °C for 60 s, and 72 °C for 90 s; followed by an extension at 72 °C for 5 min. *RAG2* amplifications used the conditions: initial denaturation at 94 °C for 120 s; followed by 40 cycles of 94 °C for 30 s, 50 °C for 60 s, and 72 °C for

120 s; followed by a final extension at 72 °C for 7 min. Sequencing was completed using internal sequencing primers ExoFwd1 (5'-GCYACCCCTCACCCGATTTTAC-3') and ExoRev1 (5'-CTTTRTATGAGAAGTAGGGGTGG-3') (*cytb*), and F16-Ch (5'-CTATTTGACCTGGAGTTTGG-3') and R17-Ch (5'-GAGTCAGAGGTCAGTGAGTG-3') (*RAG2*). Sequences were examined, edited, and aligned using Sequencher, version 4.6 (Gene Codes Corporation).

PHYLOGENETIC ANALYSIS

Maximum parsimony (MP) analyses were conducted using the combined evidence dataset (both genes), as well as for *cytb* and *RAG2* separately. Saturation analyses indicated that inclusion of *cytb* third positions was appropriate. Heuristic searches were implemented using PAUP*, version 4.10b (Swofford, 2000), with tree bisection–reconnection (TBR) branch swapping and 10 000 random taxon addition replicates. MP bootstrap analyses were performed using an equally weighted heuristic search with 1000 replicates, 100 addition sequence replicates, and TBR branch swapping. For all analyses, the outgroup taxon *Zenarchopterus buffonis* was used to root phylogenetic trees.

For Bayesian analysis, MrModeltest, version 2.3 (Nylander, 2004) was used to select models of evolution based on Akaike information criteria (Posada & Buckley, 2004). MrModeltest was run on the combined dataset, as well as *cytb* and *RAG2* separately. Bayesian Inference (BI) analyses were performed using MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003) and were conducted on the combined evidence dataset (both genes) as well as for *cytb* and *RAG2* separately. For all analyses, convergence between concurrent runs was assessed by PSRF values approaching 1.000 and an even distribution of posterior probabilities (Ronquist & Huelsenbeck, 2003). A general time reversible model with invariable sites and a gamma shaped distribution (GTR+I+Γ) was applied and run for 20 million generations (sampling every 1000 trees and discarding 25% as burn-in). For the combined dataset, the 'unlink' command was used to estimate parameters independently for each gene.

RECONSTRUCTING THE EVOLUTION OF GLIDING STRATEGIES

To reconstruct the evolution of gliding in flyingfishes, gliding strategy was categorized as a multistate character and optimized on our trees using the 'trace' command in MacClade, version 4.07 (Maddison & Maddison, 2005). Breder's (1930) proposed distinction

Table 1. Data for specimens used in the present study, voucher catalogue information, collection localities, and GenBank accession numbers

Taxon	Museum catalogue number	Collection locality	GenBank number (cytb)	GenBank number (RAG2)
<i>Cheilopogon abei</i>	No voucher	Indian Ocean	HQ325604	HQ325671
<i>Cheilopogon abei</i>	ROM-79328	Eastern Tropical Pacific	HQ325605	HQ325672
<i>Cheilopogon atrisignis</i>	No voucher	Eastern Tropical Pacific	HQ325606	HQ325673
<i>Cheilopogon atrisignis</i>	SIO-07-142	Eastern Tropical Pacific	HQ325607	HQ325674
<i>Cheilopogon cyanopterus</i>	SIO-07-145	Indian Ocean	HQ325608	HQ325675
<i>Cheilopogon cyanopterus</i>	SIO-07-145	Indian Ocean	HQ325609	HQ325676
<i>Cheilopogon dorsomacula</i>	SIO-07-139	Eastern Tropical Pacific	HQ325610	HQ325677
<i>Cheilopogon dorsomacula</i>	SIO-07-140	Eastern Tropical Pacific	HQ325611	HQ325678
<i>Cheilopogon exsiliens</i>	SIO-07-143	Atlantic	HQ325612	HQ325679
<i>Cheilopogon exsiliens</i>	SIO-07-143	Atlantic	HQ325613	HQ325680
<i>Cheilopogon furcatus</i>	ROM-79317	Gulf of Mexico	HQ325614	HQ325681
<i>Cheilopogon furcatus</i>	ROM-79259	Gulf of Mexico	HQ325615	HQ325682
<i>Cheilopogon melanurus</i>	UF-99877	Gulf of Mexico	HQ325616	HQ325683
<i>Cheilopogon melanurus</i>	UF-99882	Gulf of Mexico	HQ325617	HQ325684
<i>Cheilopogon pinnatibarbatus (californicus)</i>	SIO-07-134	Eastern Tropical Pacific	HQ325618	HQ325685
<i>Cheilopogon spilonotopterus</i>	SIO-07-127	Eastern Tropical Pacific	HQ325619	HQ325686
<i>Cheilopogon spilonotopterus</i>	SIO-07-137	Eastern Tropical Pacific	HQ325620	HQ325687
<i>Cheilopogon xenopterus</i>	ROM-79248	Eastern Tropical Pacific	HQ325621	HQ325688
<i>Cheilopogon xenopterus</i>	ROM-79248	Eastern Tropical Pacific	HQ325622	HQ325689
<i>Cypselurus angusticeps</i>	SIO-07-141	Eastern Tropical Pacific	HQ325623	HQ325690
<i>Cypselurus angusticeps</i>	SIO-07-141	Eastern Tropical Pacific	HQ325624	HQ325691
<i>Cypselurus callopterus</i>	SIO-07-131	Eastern Tropical Pacific	HQ325625	HQ325692
<i>Cypselurus callopterus</i>	SIO-07-131	Eastern Tropical Pacific	HQ325626	HQ325693
<i>Cypselurus hexazona</i>	SAMAF9778	Indo-Pacific	HQ325627	HQ325694
<i>Exocoetus monocirrhus</i>	SIO-07-129	Eastern Tropical Pacific	HQ325628	HQ325695
<i>Exocoetus monocirrhus</i>	ROM-79270	Eastern Tropical Pacific	HQ325629	HQ325696
<i>Exocoetus obtusirostris</i>	USNM-380590	Atlantic	HQ325630	HQ325697
<i>Exocoetus obtusirostris</i>	USNM-380574	Atlantic	HQ325631	HQ325698
<i>Exocoetus peruvianus</i>	SIO-07-125	Eastern Tropical Pacific	HQ325632	HQ325699
<i>Exocoetus peruvianus</i>	SIO-07-125	Eastern Tropical Pacific	HQ325633	HQ325700
<i>Exocoetus volitans</i>	SIO-07-132	Eastern Tropical Pacific	HQ325634	HQ325701
<i>Exocoetus volitans</i>	SIO-07-132	Eastern Tropical Pacific	HQ325635	HQ325702
<i>Exocoetus volitans</i>	USNM-380582	Atlantic	HQ325636	HQ325703
<i>Exocoetus volitans</i>	USNM-380581	Atlantic	HQ325637	HQ325704
<i>Fodiator rostratus</i>	SIO-07-128	Eastern Tropical Pacific	HQ325638	HQ325705
<i>Fodiator rostratus</i>	SIO-07-128	Eastern Tropical Pacific	HQ325639	HQ325706
<i>Hemiramphus far</i>	ZRC-40625	Singapore	AY693516.1	AY693582.1
<i>Hemiramphus far</i>	ZRC-40625	Singapore	AY693517.1	AY693583.1
<i>Hirundichthys affinis</i>	USNM-380592	Atlantic	HQ325640	HQ325707
<i>Hirundichthys affinis</i>	USNM-380588	Atlantic	HQ325641	HQ325708
<i>Hirundichthys affinis</i>	ROM-79329	Gulf of Mexico	HQ325642	HQ325709
<i>Hirundichthys albimaculatus</i>	SIO-07-126	Eastern Tropical Pacific	HQ325643	HQ325710
<i>Hirundichthys marginatus</i>	ROM-79330	Eastern Tropical Pacific	HQ325644	HQ325711
<i>Hirundichthys marginatus</i>	ROM-79205	Eastern Tropical Pacific	HQ325645	HQ325712
<i>Hirundichthys rondeletii (volador)</i>	ROM-79252	Gulf of Mexico	HQ325646	HQ325713
<i>Hirundichthys rondeletii (volador)</i>	ROM-79273	Gulf of Mexico	HQ325647	HQ325714
<i>Hirundichthys rondeletii (volador)</i>	ROM-79265	Gulf of Mexico	HQ325648	HQ325715
<i>Hirundichthys rondeletii (volador)</i>	ROM-79324	Gulf of Mexico	HQ325649	HQ325716
<i>Hirundichthys rondeletii (volador)</i>	ROM-79290	Gulf of Mexico	HQ325650	HQ325717
<i>Hirundichthys speculiger</i>	SIO-07-133	Eastern Tropical Pacific	HQ325651	HQ325718
<i>Hirundichthys speculiger</i>	SIO-07-137	Eastern Tropical Pacific	HQ325652	HQ325719

Table 1. Continued

Taxon	Museum catalogue number	Collection locality	GenBank number (cytb)	GenBank number (RAG2)
<i>Hirundichthys speculiger</i>	SIO-07-144	Indo-Pacific (Taiwan)	HQ325653	HQ325720
<i>Hyporhamphus quoyi</i>	ZRC-40626	Singapore	AF243919.1	AY693551.1
<i>Hyporhamphus quoyi</i>	ZRC-40626	Singapore	AF243920.1	AY693552.1
<i>Oxyporhamphus micropterus</i>	No voucher	Eastern Tropical Pacific	AY693489.1	AY693560.1
<i>Oxyporhamphus micropterus</i>	No voucher	Eastern Tropical Pacific	AY693490.1	AY693561.1
<i>Oxyporhamphus micropterus (similis)</i>	USNM-380572	Atlantic	HQ325654	HQ325721
<i>Oxyporhamphus micropterus (similis)</i>	USNM-380573	Atlantic	HQ325655	HQ325722
<i>Parexocoetus brachypterus</i>	ROM-79331	Eastern Tropical Pacific	HQ325656	HQ325723
<i>Parexocoetus brachypterus</i>	ROM-79312	Pacific	HQ325657	HQ325724
<i>Parexocoetus hillianus</i>	UF-99876	Gulf of Mexico	HQ325658	HQ325725
<i>Parexocoetus hillianus</i>	UF-99883	Gulf of Mexico	HQ325659	HQ325726
<i>Parexocoetus mento</i>	No voucher	Pacific	HQ325660	HQ325727
<i>Parexocoetus mento</i>	S-16008-001	Indo-Pacific	HQ325661	HQ325728
<i>Prognichthys gibbifrons</i>	SIO-07-143	Gulf of Mexico	HQ325662	HQ325729
<i>Prognichthys gibbifrons</i>	ROM-79332	Gulf of Mexico	HQ325663	No sequence
<i>Prognichthys glaphyrae</i>	ROM-79333	Gulf of Mexico	HQ325664	HQ325730
<i>Prognichthys glaphyrae</i>	ROM-79334	Unknown	HQ325665	HQ325731
<i>Prognichthys occidentalis</i>	ROM-79291	Gulf of Mexico	HQ325666	HQ325732
<i>Prognichthys sealei</i>	SIO-07-130	Eastern Tropical Pacific	HQ325667	HQ325733
<i>Prognichthys sealei</i>	SIO-07-130	Eastern Tropical Pacific	HQ325668	HQ325734
<i>Prognichthys tringa</i>	SIO-07-135	Gulf of California	HQ325669	HQ325735
<i>Prognichthys tringa</i>	SIO-07-138	Eastern Tropical Pacific	HQ325670	HQ325736
<i>Zenarchopterus buffonis</i>	CU-77844	Bunaken, Sulawesi	AF243921.1	AY693553.1
<i>Zenarchopterus buffonis</i>	CU-77844	Bunaken, Sulawesi	AF243922.1	AY693554.1

cytb, cytochrome *b*; RAG2, recombination activating gene 2.

between two-wing and four-wing gliding in flyingfishes has largely been followed in the literature (Fish, 1990; Davenport, 1994); thus, gliding strategy was coded as a multistate character with the states 'absent', 'two-wing', and 'four-wing'. The elongated dorsal fin of *Parexocoetus* may serve as an additional gliding surface, resulting in three-wing gliding (R. L. Pitman, pers. observ.); however, this phenomenon has not been formally described and thus will not be specifically addressed in the present study (see below).

RANGE SIZE AND LIFE-HISTORY CHARACTERS

To test whether flyingfish species range size is correlated with certain life history characters, we used the concentrated-changes test (CCT) of Maddison (1990; see Maddison & Maddison, 1992) as implemented by MacClade, version 4.07 (Maddison & Maddison, 2005). The CCT determines whether changes in a particular character (the dependent character) are concentrated on branches that have a specified state for a second character (the independent character). Specifically, we tested the macroecological predictions

that: (1) flyingfishes with buoyant eggs have larger geographic ranges than those with nonbuoyant eggs and (2) flyingfishes that complete their entire lifecycle far offshore have larger geographic ranges than those that include an inshore component to their lifecycle.

Data on egg buoyancy, habitat, and geographic range size were determined from the literature (see Supporting information, Table S1) and coded as binary characters. Each species in our phylogeny was coded as having eggs that are either nonbuoyant (0) or buoyant (1), habitat preference that is either meroepipelagic (0) or holoeipelagic (1), and a range size that is either limited to a single ocean (0) or spans multiple oceans (1). We defined the two habitat states based on Parin's (1968) work, where meroepipelagic species are defined as using coastal (continental shelf) waters during some period of their lives, whereas holoeipelagic species are defined as taxa that complete all life stages in the open ocean (off of the continental shelf). We use the presence of a species in either one or multiple oceans as a coarse proxy for more precise measurements of species range size because other procedures, such as digitizing areas from maps, could not be completed for a

reasonable number of species (i.e. the majority of exocoetids). Pending improved biogeographic data for flyingfish species, we consider that this approximation allows reasonable, albeit conservative, tests of our hypotheses.

To implement the CCT, redundant operational taxonomic units (multiple representatives of the same species) were pruned from trees and polytomies were resolved manually (a necessity for the test). Characters were optimized with equivocal reconstructions resolved using both DELTRAN and ACCTRAN, however, the DELTRAN method was preferred because it does not force an increase in the number of observed character reversals (Maddison & Maddison, 2005).

RESULTS

PHYLOGENETIC RELATIONSHIPS

A total of 2019 bp were amplified and sequenced from the mitochondrial cytb gene (1137 bp) and the nuclear RAG2 gene (882 bp). Of these, 1289 characters were constant, 51 were variable but parsimony uninformative, and 679 were parsimony informative. MP analyses yielded 1059 equally parsimonious trees of 2464 steps each, and a strict consensus is shown in Figure 2. Most nodes are well-supported, with 45 of 60 nodes having BS > 80. The family Exocoetidae, excluding *Oxyporhamphus*, is found to be monophyletic. Also, recognized subfamily, genus, and species-level groupings were generally monophyletic. An exception is the genus *Cheilopogon*, which was divided into two well-supported clades, named here *Cheilopogon* Clade A and *Cheilopogon* Clade B. Separate analyses of cytb and RAG2 (not shown) produced results that were largely congruent with the combined evidence trees. The BI combined evidence analysis produced phylogenetic reconstructions that were largely consistent with MP; however, less resolution was observed for some major clades (Fig. 3). There were some differences between the MP and BI trees. Both analyses strongly supported the monophyly of flyingfishes but MP positioned *Fodiator* as the sister group to all other flyingfishes, whereas BI showed *Parexocoetus* in that position. By contrast to MP, BI failed to provide evidence for a monophyletic Cypselurinae (*Prognichthys*, *Cypselurus*, *Cheilopogon*, and *Hirundichthys*), and also failed to support the monophyly of *Hirundichthys*. Finally, MP showed *Cheilopogon* clade B (see below) as the sister group of all other *Cheilopogon* + *Cypselurus* + *Prognichthys*, whereas BI placed *Cheilopogon* clade A in that position. In general, BI nodes that conflicted with the MP results showed relatively low posterior probabilities.

When compared with BI, our combined MP analysis was better resolved, and more congruent with previous morphology-based phylogenies (Parin, 1961; Collette *et al.*, 1984; Dasilao & Sasaki, 1998). Our MP results agree with morphology in placing *Fodiator* as the basal flyingfish lineage rather than *Parexocoetus* (Figs 1, 2). Also, MP supports the monophyly of Cypselurinae, a node supported by several anatomical synapomorphies (Collette *et al.*, 1984; Dasilao & Sasaki, 1998). Given the congruence between our combined MP analysis and previous morphological investigations, as well as the higher resolution of the MP analysis, we use the combined MP tree as our preferred hypothesis of flyingfish relationships.

FLYINGFISH GLIDING

Our finding of a monophyletic Exocoetidae supports the idea that true gliding evolved a single time in this group. The earliest condition within flyingfishes, based on character optimization, is the two-wing state (exhibited by *Fodiator*, *Parexocoetus*, and *Exocoetus*) (Fig. 4). Four-wing gliding had a single origin within Cypselurinae, and is relatively derived (Fig. 4).

EGG BUOYANCY, HABITAT PREFERENCE, AND RANGE SIZE

Figure 5 summarizes the optimization of egg buoyancy, habitat preference, and range size characters on our preferred flyingfish species phylogeny. For egg buoyancy, the plesiomorphic condition is nonbuoyant eggs, with buoyant eggs evolving multiple times: once in *Exocoetus*, once in *Prognichthys*, and one or more times in *Cheilopogon* clade A. For habitat preference, the plesiomorphic condition is meroepipelagic, and the holoeipelagic state has evolved in several clades, including *Exocoetus*, *Hirundichthys*, *Cheilopogon furcatus*, *Prognichthys*, and *Cheilopogon* clade A. Range size exhibits a complex pattern of evolution, with eight bidirectional changes between the restricted (single ocean) and widespread (two or more oceans) states.

Using the CCT, we were unable to reject the null hypothesis that large species ranges (occupying two or more oceans) have evolved randomly with respect to lineages that exhibit buoyant eggs (CCT *P*-value = 0.13). Thus, having buoyant eggs does not appear to affect the evolution of flyingfish species ranges. However, CCT did reject the null hypothesis that large species ranges have evolved randomly with respect to lineages that are holoeipelagic (CCT *P*-value = 0.0007). This indicates that large range sizes are more likely to evolve in lineages with flyingfish species that are holoeipelagic.

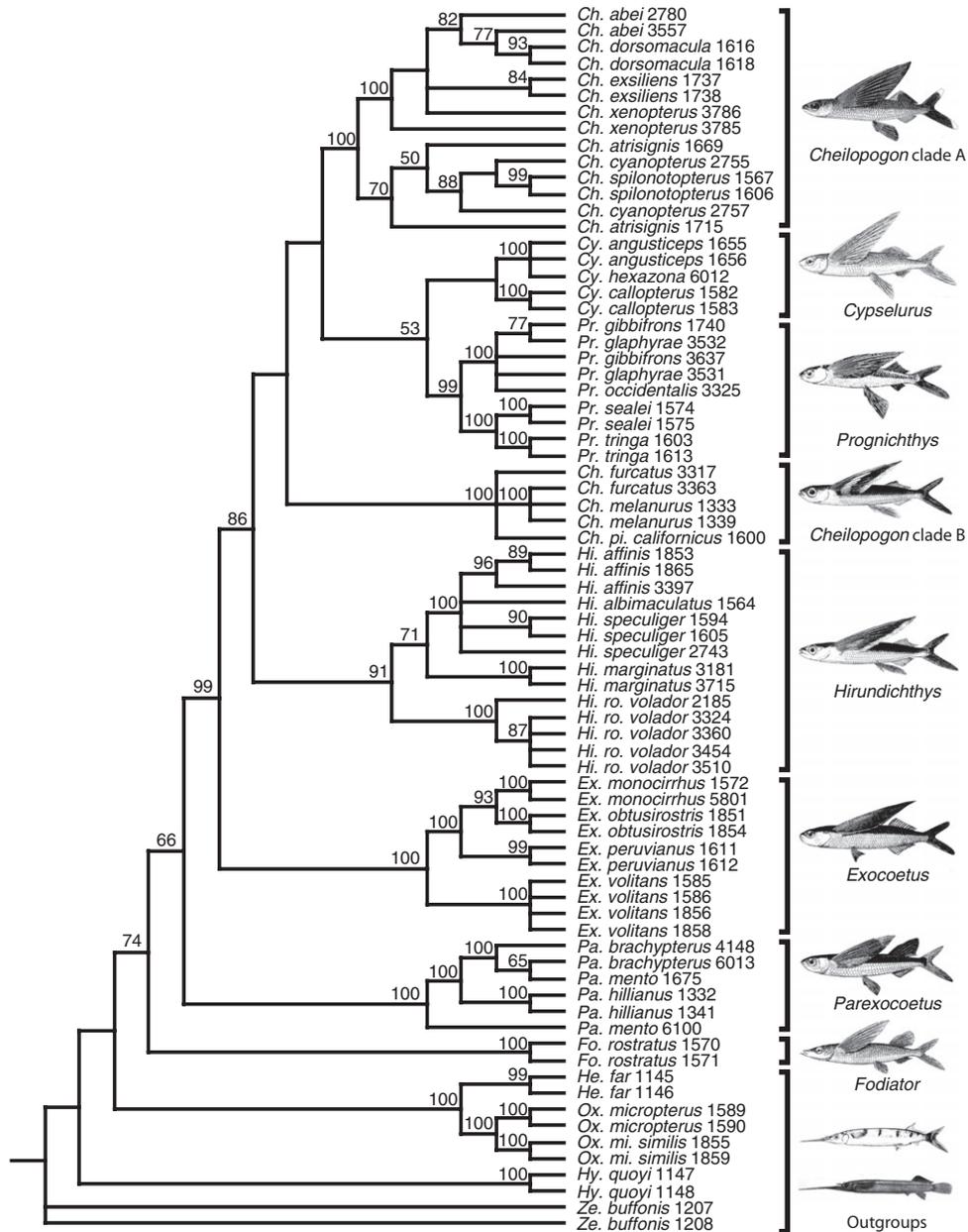


Figure 2. Cladogram of the strict consensus of 1059 most parsimonious trees based on combined parsimony analysis of cytochrome *b* (*cytb*) and recombination activating gene 2 (*RAG2*) sequence data. Numbers above nodes are bootstrap proportions. The names (and references) of species illustrations used to represent each clade are listed here. From top: *Cheilopogon abei*, *Cypselurus angusticeps*, *Prognichthys sealei*, *Cheilopogon furcatus*, *Hirundichthys speculiger*, *Exocoetus monocirrhus*, *Parexocoetus mento* (Parin, 1999); *Fodiator rostratus* (Parin, 1995); *Hemiramphus far* (Collette, 1999); *Zenarchopterus buffonis* (Froese & Pauly, 2010).

DISCUSSION

PHYLOGENY OF FLYINGFISHES

As in previous studies (Parin, 1961; Collette *et al.*, 1984; Dasilao & Sasaki, 1998), we found strong support for the monophyly of flyingfishes. We also found molecular support for the monophyly of each of

the four subfamilies (Fodiatorinae, Parexocoetinae, Exocoetinae, and Cypselurinae) and the previously proposed pattern of phylogenetic relationships among these subfamilies (Bruun, 1935; Parin, 1961; Collette *et al.*, 1984; Dasilao & Sasaki, 1998). By contrast to Dasilao *et al.* (1997), who proposed a sister group relationship between the traditionally recognized

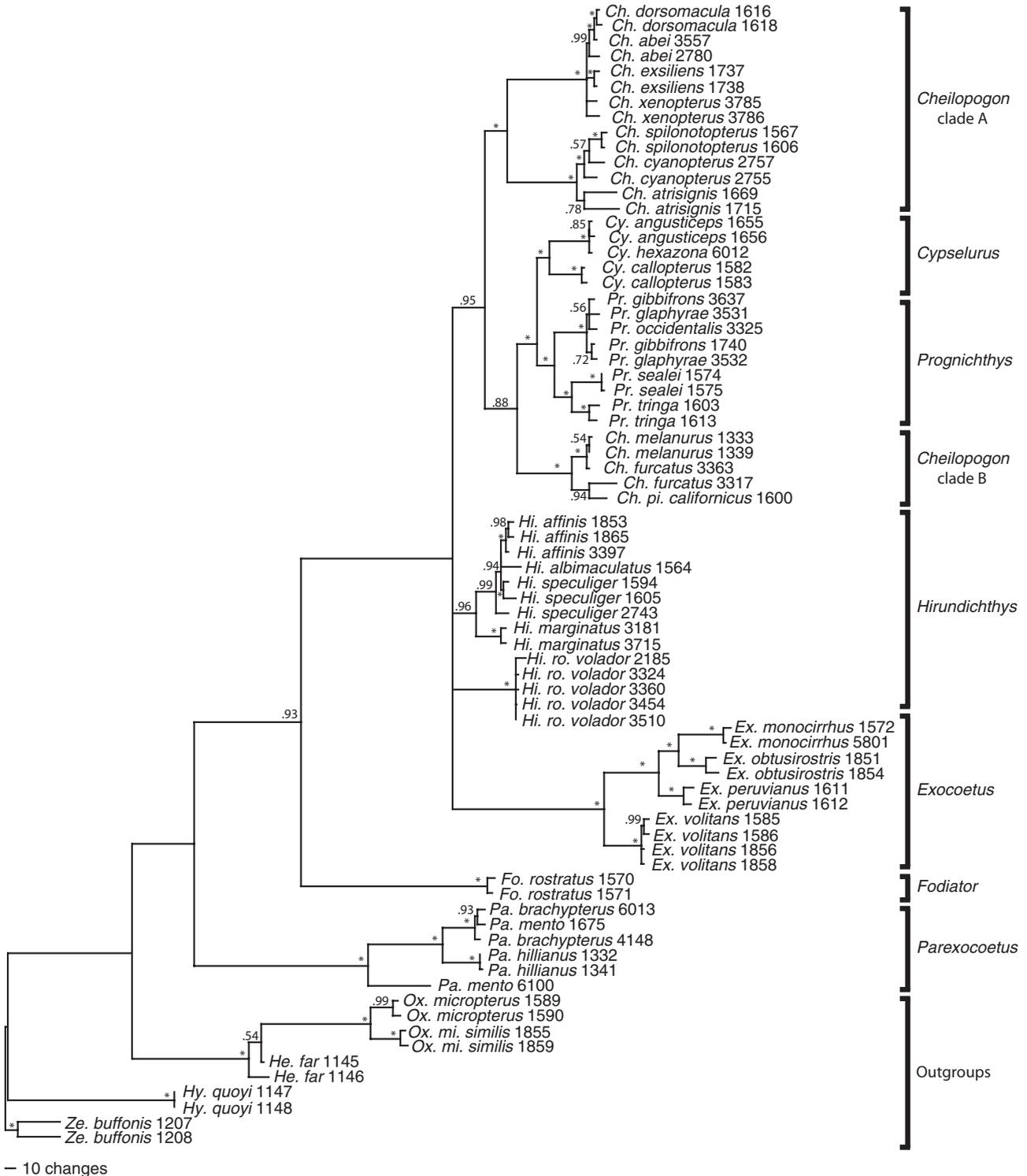


Figure 3. Phylogram from combined Bayesian analysis of cytochrome *b* (cytb) and recombination activating gene 2 (RAG2) sequence data. Numbers above nodes indicate Bayesian posterior probabilities (* = 1.00).

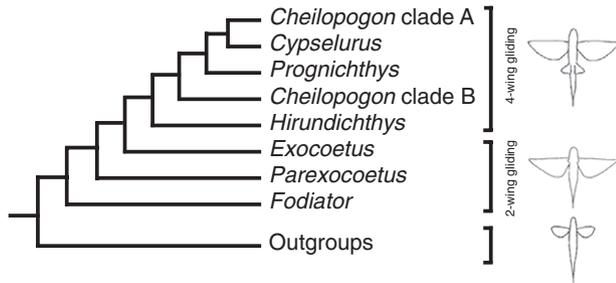


Figure 4. Genus level phylogenetic hypothesis for flyingfishes, simplified from maximum parsimony analysis of the full dataset (see Figure 2), and showing the evolution of gliding strategies. Gliding illustrations *sensu* Davenport (2003).

Exocoetidae and the genus *Oxyporhamphus*, the present study confirms the result of Lovejoy *et al.* (2004) in placing *Oxyporhamphus* with *Hemiramphus*. This finding has implications for reconstructions of the earliest evolution of gliding.

Our molecular results support the monophyly of most currently recognized flyingfish genera. In general, our analyses included high proportions of the recognized species within each genus; we included five of six recognized species of *Prognichthys*, five of eight *Hirundichthys*, four of five *Exocoetus*, three of three *Parexocoetus*, and one of two *Fodiator* (numbers of recognized species from Froese & Pauly, 2010). This level of sampling strengthens our case of the monophyly of these genera. Our taxon sampling was weaker for the more diverse genera *Cypselurus* (three of ten recognized species included) and *Cheilopogon* (ten of 33 recognized species included) (Parin, 2009; Froese & Pauly, 2010). Our results indicate that the latter genus, the most diverse and morphologically variable within flyingfishes, is not monophyletic. Support for this result was high, based on consistency across analyses and bootstrap and posterior probability values. Several *Cheilopogon* subgenera have been proposed (Parin, 1961), although our limited species sampling for this genus makes it difficult to determine how well our clade A and clade B correspond to these subgeneric designations.

Several studies have questioned whether *Cypselurus* and *Cheilopogon* are distinct genera (Bruun, 1935; Staiger, 1965; Gibbs & Staiger, 1970; Dasilao & Sasaki, 1998). However, our molecular results support the contention of Parin (1961) and Collette *et al.* (1984) that these are distinct taxa, with *Cypselurus* more closely related to *Prognichthys* than it is to either of the *Cheilopogon* clades.

EVOLUTION OF FLYINGFISH GLIDING STRATEGIES

The gliding behaviours of flyingfishes have long been of interest to evolutionary biologists (Darwin, 1872;

Möbius, 1878; Dunford, 1906; Breder, 1930; Fish, 1990; Davenport, 1994; for a review, see Kutschera, 2005). Exocoetids exhibit a range of gliding capabilities, from weak gliders like *Fodiator*, to the two-winged *Exocoetus* that glide short distances (tens of metres), to the four-winged Cypselurinae that can glide hundreds of metres (Fish, 1990; Davenport, 1994). The evolutionary trajectory of gliding in flyingfishes has been discussed (Dasilao & Sasaki, 1998; Kutschera, 2005) and most recent studies have concluded that a progressive evolution of gliding took place, from two-wing to four-wing (Parin, 1961; Collette *et al.*, 1984; Dasilao *et al.*, 1997; Dasilao & Sasaki, 1998). The results of the present study support the hypothesis that two-winged (two-wing) gliding evolved first in flyingfishes and four-winged (four-wing) gliding evolved more recently.

Although gliding has frequently and traditionally been considered a two state character (two-wing and four-wing), both anatomical and functional analyses suggest a more complex pattern of evolution. Dasilao & Sasaki (1998) presented a detailed reconstruction of the evolution of anatomical features associated with gliding. Their scenario describes a progression, with the following characters added sequentially: (1) enlarged pectoral fins and associated muscles at the exocoetid node; (2) more greatly enlarged pectoral fins at the *Exocoetus* + Cypselurinae node; and (3) enlarged pelvic fins at the Cypselurinae node. This scenario would thus define three groups characterized by different suites of morphological features related to gliding. Data collected by Fish (1990) lend support to this idea. Fish (1990) measured body mass, wing area, and tail area for several flyingfish genera, and calculated the aerodynamic parameters of wing loading and aspect ratio. He found differences between the three groups described above in a combination of characteristics, including % wing area composed of pectoral fin, deviations (or lack thereof) from geometric scaling for wing span and wing area, and wing aspect ratio (Fish, 1990). The molecular phylogeny presented here agrees with the three-step scenario of gliding evolution. However, we suggest that further phylogenetic optimizations of detailed functional characters, such as the very high wing aspect ratio in *Exocoetus* (Fish, 1990), and the use of the laterally inclined dorsal fin as a gliding surface in *Parexocoetus* (R. L. Pitman, pers. observ.), represent autapomorphies that deserve further investigation.

Reconstructing the earliest origin of gliding behaviour and anatomy in the Exocoetidae will depend on an accurate assessment of the family's nearest relatives. Dasilao *et al.* (1997) placed *Oxyporhamphus* as the sister group to flyingfishes, based on morphology. *Oxyporhamphus* is a genus of two epipelagic species that exhibit limited jumping and gliding behaviour

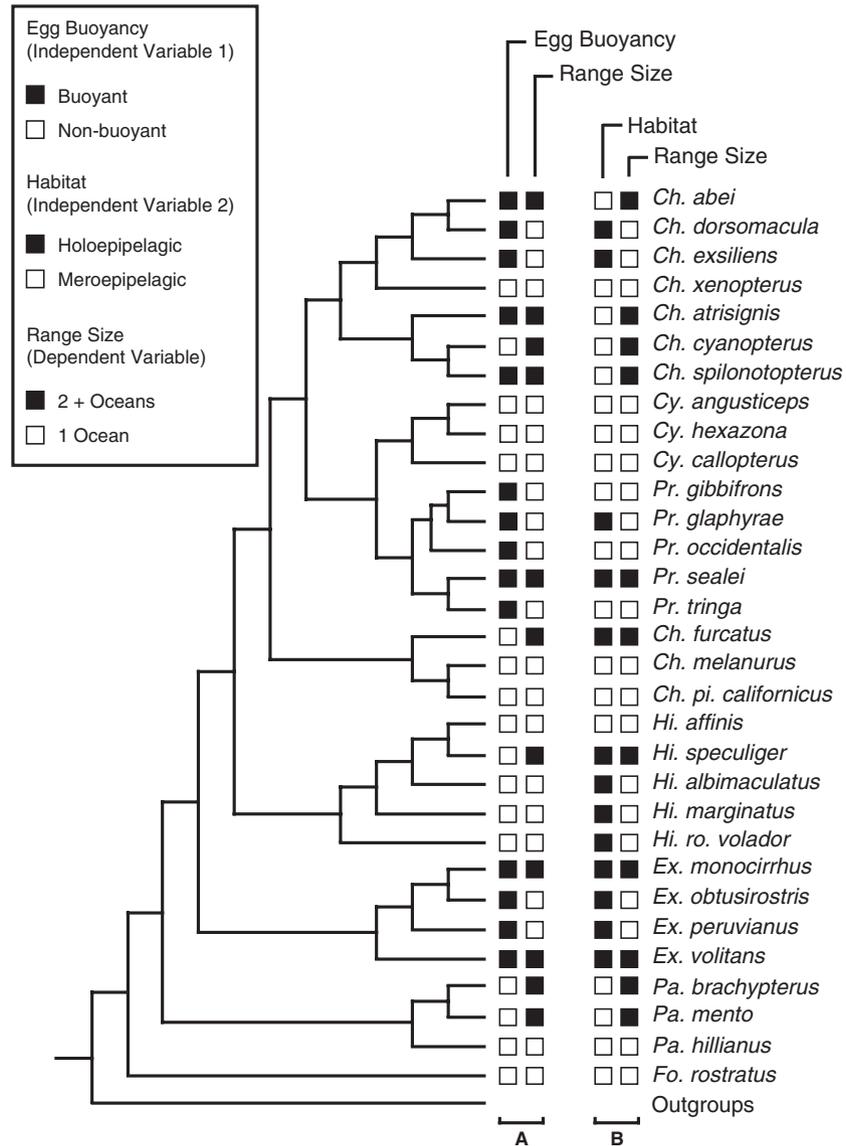


Figure 5. Character optimizations (on parsimony tree) used to conduct concentrated changes tests (CCT). Multiple representatives of the same species have been pruned. (A) egg buoyancy (independent character) and species range size (dependent character). (B) habitat (independent character) and species range size (dependent character). Range size shown twice to facilitate comparison with the two independent characters. Closed squares represent presence, and open squares represent absence of characters.

and have been grouped with either halfbeaks (Gill, 1864; Regan, 1911; Bruun, 1935; Parin, 1961; Norman, 1966; Collette *et al.*, 1984; Collette, 2004) or flyingfishes (Nichols & Breder, 1928; Hubbs, 1935; Dasilao *et al.*, 1997). In contrast with Dasilao *et al.* (1997), molecular studies place *Oxyporhamphus* with *Hemiramphus* (Lovejoy *et al.*, 2004; present study). Swim bladder morphology provides additional evidence for the latter relationship (Tibbetts *et al.*, 2007). The placement of *Oxyporhamphus* away from the flyingfishes suggests that some aspects of gliding

behaviour and anatomy have evolved independently within beloniform fishes. However, resolution of this issue depends on an analysis with more extensive sampling of halfbeaks, particularly *Hemiramphus*.

The selective pressures responsible for the origin and elaboration of gliding in flyingfishes remain unresolved. The consensus is that predator avoidance is the most reasonable explanation (Mohr, 1954; Evans & Sharma, 1963; Fish, 1990; Gillett & Ianelli, 1991; Davenport, 1992; Davenport, 1994; Kutschera, 2005). Flyingfishes share epipelagic habitats with

high-speed predators such as billfishes (Istiophoridae), dolphinfishes (Coryphaenidae), tunas (Scombridae), and dolphins (e.g. *Stenella* spp.), and diet analyses suggest that these taxa feed on exocoetids (Olson, 1982; Olson & Boggs, 1986; Richard & Barbeau, 1994; Oxenford & Hunte, 1999). Unlike reef or shore habitats, which offer natural cover for fishes, flyingfish habitat is largely refuge free. Thus, mechanisms of predator evasion are at a premium, and the predator avoidance hypothesis posits that gliding evolved as a means of escaping a suddenly hostile environment. This historical hypothesis is difficult to test; however, it might be possible to test for correlations between the presence of particular types of predators and types of gliding behaviour. For example, field observations (R. L. Pitman, pers. observ.), indicate that *Exocoetus* co-occurs with tunas, whereas Cypselurinae individuals are frequently the prey of dolphinfishes. Tunas (Scombridae) hunt in large, fast-swimming schools, and may be avoided best by two-wing flyingfishes that are able to exit the water quickly and without the need of a 'taxiing' phase (Hubbs, 1935; Fish, 1990). On the other hand, dolphinfishes (Coryphaenidae) actively pursue prey (Davenport, 1994) and may be evaded best by the longer glides, faster speeds, and abrupt changes in direction achieved by four-wing flyingfish species. Habitat modeling of these predator/prey systems could provide tests of these hypotheses.

EGG BUOYANCY, HABITAT, AND SPECIES RANGE SIZE

Our analyses using CCT suggest that flyingfish habitat preference, but not egg buoyancy, has an effect on the species range size. We tested the effects of these particular characters because they were obtainable for a reasonable number of species from the literature, and because both habitat selection and dispersal ability have been correlated with range size (e.g. Böhning-Gaese *et al.*, 2006). It can be argued that range size is not heritable over evolutionary time, and thus not appropriate for phylogenetic tests such as the CCT (Webb & Gaston, 2003; Kunin, 2008); however, the position that range size is a heritable character has also been defended (Hunt, Roy & Jablonski, 2005; Waldron, 2007), and phylogenetic methods incorporating range size have been used previously (Rundle *et al.*, 2007).

Within flyingfishes, nonbuoyant eggs covered with evenly distributed filaments are plesiomorphic. These eggs are usually attached to vegetation or other floating objects. Some taxa, such as *Exocoetus*, have evolved buoyant eggs without filaments that apparently require no substrate for egg attachment. We predicted that buoyant eggs would facilitate long-distance dispersal, as has been observed in other

marine taxa that produce pelagic eggs (Bradbury *et al.*, 2008), and would be correlated with increased range size. However, our analysis did not show a positive effect of buoyant eggs on the evolution of large species ranges. This could be a result of the conservative nature of the test and character coding (see below), the confounding effects of other life-history characteristics (such as duration of planktonic larval stages), or perhaps the movement of floating vegetation is itself an effective dispersal mechanism. Mats of algae and floating material are known to facilitate dispersal in marine fishes (Mora, Francisco & Zapata, 2001), invertebrates (Highsmith, 1985), and plants (Minchinton, 2006) and could feasibly affect the dispersal ability (and decrease the importance of buoyant eggs) of flyingfish species that possess filamentous eggs.

By contrast, the habitat occupied by flyingfish species did appear to positively affect the evolution of species range size. Species and lineages that were characterized as holoeipipelagic (i.e. that complete their entire lifecycle offshore) were found to evolve large ranges more frequently than species or lineages that were classed as meroeipipelagic (i.e. that have an inshore component to their lifecycle). A similar pattern was observed by Robertson, Grove & McCosker (2004) who showed that fishes from epipelagic habitats usually had distributions that stretched across the entire Pacific Ocean, while fewer inshore pelagic or demersal fishes showed the same extent of distribution (see also; Mora & Robertson, 2005; Macpherson & Raventos, 2006). Our results support the idea that species habitat has a macroecological effect on the evolution of range size.

Our approach relies on ocean basins as a proxy for distribution size, and this may have limited our ability to distinguish an effect of egg buoyancy. Species distribution size results from complex interactions between multiple factors, including organism characteristics, phylogenetic history, and environmental conditions (Böhning-Gaese *et al.*, 2006; Gaston, 2009), and future investigations on flyingfishes could incorporate more of these potentially relevant variables.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Species, gliding strategies, species distributions, geographic range sizes, habitat, and egg buoyancy characteristics used for concentrated changes tests.

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