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Repeated invasions into the twilight zone: evolutionary origins of a novel assemblage of fishes from deep Caribbean reefs

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Abstract

Mesophotic and deeper reefs of the tropics are poorly known and underexplored ecosystems worldwide. Collectively referred to as the 'twilight zone', depths below ~30–50 m are home to many species of reef fishes that are absent from shallower depths, including many undescribed and endemic species. We currently lack even a basic understanding of the diversity and evolutionary origins of fishes on tropical mesophotic reefs. Recent submersible collections in the Caribbean have provided new specimens that are enabling phylogenetic reconstructions that incorporate deep-reef representatives of tropical fish genera. Here, we investigate evolutionary depth transitions in the family Gobiidae (gobies), the most diverse group of tropical marine fishes. Using divergence-time estimation coupled with stochastic character mapping to infer the timing of shallow-to-deep habitat transitions in gobies, we demonstrate at least four transitions from shallow to mesophotic depths. Habitat transitions occurred in two broad time periods (Miocene, Pliocene–Pleistocene), and may have been linked to the availability of underutilized niches, as well as the evolution of morphological/behavioural adaptations for life on deep reefs. Further, our analysis shows that at least three evolutionary lineages that invaded deep habitats subsequently underwent speciation, reflecting another unique mode of radiation within the Gobiidae. Lastly, we synthesize depth distributions for 95 species of Caribbean gobies, which reveal major bathymetric faunal breaks at the boundary between euphotic and mesophotic reefs. Ultimately, our study is the first rigorous investigation into the origin of Caribbean deep-reef fishes and provides a framework for future studies that utilize rare, deep-reef specimens.

Keywords: adaptive radiation, Deep Reef Observation Project, Gobiidae, mesophotic reefs, phylogenetic comparative methods, phylogeny, speciation

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Introduction

Exploration of Caribbean deep-reef fishes

Shallow reefs in the Caribbean have been extensively surveyed over the last half-century, largely due to the widespread availability of scuba diving technology beginning in the 1950s. As a result, the diversity and taxonomic structure of assemblages of reef fishes on Caribbean reefs living shallower than ~50 m are well characterized (e.g. Böhlke & Chaplin 1993; Smith & Knopf 1997; Floeter *et al.* 2008; Humann & Deloach 2014; Robertson & Van Tassell 2015). In contrast, much more limited information is available on reef-fish assemblages from the twilight zone—that is mesophotic

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(~30–150 m, see Bongaerts *et al.* 2015) and deeper reefs that extend down to 300 m or beyond in the same region. Our current understanding of western Atlantic fish communities from the twilight zone comes primarily from a limited number of submersible observations (Colin 1974, 1976 [depth range sampled = DRS 50–305 m]; Nelson & Appeldoorn 1985 [DRS 50–915 m]; Parker & Ross 1986 [DRS 23–152 m]; Dennis & Bright 1988 [DRS 20–140 m]; Itzkowitz *et al.* 1991 [DRS 25–100 m]; Ross & Quattrini 2007 [DRS 366–783 m]; unpublished Johnson Sea-link Expeditions by Harbor Branch Institute of Oceanography [DRS 20-915 m]), visual surveys by remote-operated vehicles (Spieler *et al.* 2007 [DRS 70–100 m]; Bryan *et al.* 2013 [DRS 50–120 m]; Rosa *et al.* 2015 [DRS 30–90 m]), trap or hook-and-line surveys (Bunkley-Williams & Williams 2004) and surveys from technical divers (Feitoza *et al.* 2005 [DRS 35–70]; Bejarano *et al.* 2011 [DRS 50–85 m], Bejarano *et al.* 2014 [DRS 30–70 m]; Garcia-Sais 2010 [DRS 15–50 m]; Garcia-Sais *et al.* 2010 [DRS 30–50 m]; Pinheiro *et al.* 2015 [DRS 45–130 m]).

There are several challenges when trying to characterize a fish community at depth using the survey methods above. The majority of the deep-reef studies above are strictly visual surveys, which often overlook the small and cryptic but ecologically important, highly speciose groups of reef fishes, notably the gobies (Gobiidae), and blennies (Blenniidae, Chaenopsidae, Labrisomidae), and other diminutive demersal fishes such as the basslets (Grammatidae), clingfishes (Gobiesocidae) and some seabasses (Serranidae). Small cryptic fishes account for a dominant fraction of overall reef-fish diversity and contribute substantially to coral-reef trophodynamics (Ackerman & Bellwood 2000, 2002; Depczynski & Bellwood 2003). Characterizing this community of cryptic species typically requires targeted collections using ichthyocides (Robertson & Smith-Vaniz 2008). While this may be possible on deep reefs with divers using closed-circuit rebreather technology (Pyle 1998, 1999, 2000), these methods are logistically challenging and require substantial training and experience to avoid the numerous safety risks associated with deep diving. Furthermore, bottom time and depth maxima are still greatly limited, long decompression times are involved, and rebreathers currently do not provide access to reefs deeper than about 150 m. Ultimately, as a result of these challenges, there have been few targeted efforts to collect cryptic, deep-reef fishes in the wider Caribbean region. Collections made by the Johnson Sea Link submersible program in the 1970s and 1980s provided some valuable specimens, but tissue samples that would facilitate molecular phylogenetic analyses, population genetic analyses, divergence-time estimation and other macro-evolutionary investigations were not obtained. Thus, we currently lack both a clear picture of the evolutionary origins of the majority of deep-reef fishes and a strong temporal understanding of how and when deep-reef fish communities evolved over geologic timescales.

Fish diversification on deep reefs: gobies as a model group

Deep-reef fish assemblages are more than just an underexplored extension of the shallow-reef ecosystem. Assemblages of benthic organisms that form fish habitat differ considerably between shallow and deep reefs in the Caribbean (Goreau & Goreau 1973; James & Ginsburg 1979; Liddell & Ohlhorst 1988; Liddell *et al.* 1997; Reed & Pomponi 1997). Specifically, the fore-reef slope shows a marked drop in species richness of scleractinian corals and calcareous green algae with increasing depth and changes in geomorphology (Goreau & Goreau 1973; Liddell & Ohlhorst 1988) and coral endosymbiont assemblages (Bongaerts *et al.* 2015). These changes coincide with an increased diversity of sponges and an increasing predominance of coralline algae with depth, which in turn provide the foundation for a unique and diverse fish community. Reef fish communities show trends with depth similar to those for scleractinian corals and calcareous green algae: species richness and abundance decrease as depth increases, frequently correlating with extent of live coral cover (Lukens 1981; Nelson & Appeldoorn 1985; Dennis & Bright 1988; Itzkowitz *et al.* 1991; but see Pinheiro *et al.* 2015). Given that those reported trends are based largely on visual surveys that, as mentioned, underestimate cryptic species diversity, and relationships between reef fishes and depth should be re-analysed when sufficient specimen-based data are available. Regardless, many species common on shallow reefs are rare or absent at mesophotic depths and beyond, with a significant fraction of mesophotic fish species belonging to a 'true deep-reef fauna'—that is a well-defined group of deep-reef specialists that are absent from shallow communities (Colin 1974, 1976; Pyle 1999, 2000; Brokovich *et al.* 2008; Garcia-Sais *et al.* 2010; Kahng *et al.* 2011; Bejarano *et al.* 2014). Much of the true deep-reef fauna consists of undescribed taxa that potentially include locally endemic species (Colin 1974, 1976; Pyle 1999, 2000).

Habitat partitioning by depth is thus an important factor structuring reef fish communities, and in this regard, the shallow-to-deep reef slopes of Caribbean islands provide a habitat-rich ecological gradient for fishes to exploit. Bathymetric niche partitioning has been observed within lineages of closely related coral reef fishes such as Greater Caribbean *Liopropoma* (seabasses) (Baldwin & Robertson 2014), *Elacatinus* (neon gobies) (Colin 1975, 2010), *Coryphopterus* (gobies) (Baldwin & Robertson 2015), as well as NE Pacific *Sebastes* (rockfishes) (Ingram 2011), and Indo-Pacific *Eviota* (dwarfgobies) (Greenfield & Randall 1999). Speciation driven by depth partitioning may be common in many other groups of reef fishes and could contribute substantially to the origins of the 'true deep-reef fauna'. Specifically, deep reefs could provide a variety of vacant niches that are available to species capable of adapting to a low-light, cool, resource-limited environment, thus opening the door for adaptive radiations of select lineages on deep reefs. We currently lack a clear understanding not only of the temporal rate of shallow-to-deep (or vice versa) evolutionary transitions, but also the extent of speciation that occurred exclusively on deep reefs following an invasion of a shallow-water lineage. As a result, it is unclear whether the deep-reef fish fauna comprises a conglomerate of species that largely represent single evolutionary offshoots of shallow-water clades, or whether some lineages successfully diversified at mesophotic depths.

For a variety of important reasons Caribbean gobies represent an ideal group to use as a model exploring these hypotheses. First, the Gobiidae comprise the most diverse family of marine fishes, and goby species exhibit a remarkable array of morphological and behavioural adaptations that have facilitated microhabitat specialization and adaptive radiations in a variety of marine and coastal freshwater habitats globally (Rüber *et al.* 2003; Yamada *et al.* 2009; Thacker & Roje 2011; Zander 2011). Second, gobies are particularly well represented on coral reefs, where they comprise a major component of overall diversity and occupy a broad array of ecological niches (Ackerman & Bellwood 2000, 2002; Herler *et al.* 2011). In the western Atlantic, there are nearly 100 species of shallow-water gobies that are associated with reef habitats (Van Tassell 2011). Third, gobies have the ability and propensity to speciate rapidly in association with microhabitat partitioning (e.g. Rüber *et al.* 2003; Tornabene *et al.* 2013), rendering them an ideal group for studying potential bathymetric habitat partitioning in deep-reef fishes. Lastly, gobies on Greater Caribbean reefs represent multiple independent phylogenetic lineages and thus provide an opportunity to explore the existence of patterns of parallel evolution.

Deep Reef Observation Project: incorporating deep-reef fishes into molecular phylogenies

Recently, the Smithsonian Institution's Deep Reef Observation Project (*DROP*) has added a wealth of new information to the growing body of knowledge on Caribbean deep-reef communities. Operating out of Substation Curaçao (http://www.substation-Cuaracao.com), *DROP* uses the manned submersible *Curasub* to explore deep reefs to 300 m off the coast of Curaçao and nearby areas in the southern Caribbean. Unlike many of the deep-reef surveys mentioned above, *DROP* effectively captures small and cryptic fishes, some of which have been the basis for descriptions of new species of deep-reef fishes (Baldwin & Robertson 2013, 2014, 2015; Baldwin & Johnson 2014; Tornabene *et al.* 2016). For example, a single dive to 198 m off the west coast of Curaçao in 2015 resulted in the capture of ~20 specimens of gobies representing six undescribed species, in addition to many other specimens of deep-reef fishes and invertebrates. Tissue samples from *Curasub* collections are now being incorporated into larger molecular phylogenies to assess the evolutionary origins of deep-reef taxa.

During a series of *DROP-Curasub* dives to depths of 300 m from 2011 to 2014, specimens of gobies were collected that represent 13 species, most of them recently described as new or awaiting description (Van Tassell *et al.* 2012; Baldwin & Robertson 2015; Tornabene *et al.* 2016) (Fig. 1). Whole specimens plus tissue samples of the specimens provide an excellent opportunity to investigate the phylogenetic relationships of these deep-reef species relative to their shallow-water goby relatives. By integrating these new species into the most comprehensive molecular phylogenetic hypotheses of the Gobiidae to date (see Agorreta *et al.* 2013), we can generate a clearer picture of the evolution of Caribbean deep-reef gobies and investigate the existence and timing of parallel invasions between mesophotic and shallow reefs across independent lineages. Here, we use multilocus sequence data to infer the phylogenetic positions of 13 species of deep-reef gobies from the western Atlantic (Table 1) and 244 species of their relatives. The resulting phylogeny is then used in combination with relaxed molecular clock methods and ancestral character-state inference to estimate the timing of speciation events and of bathymetric habitat transitions in gobies, and to assess whether lineages are diversifying following a transition to deep reefs. In addition, we synthesize depth distribution data for all Caribbean reef gobies to reveal bathymetric faunal breaks associated with the boundary between the euphotic and mesophotic zones, and test the hypothesis that fish diversity decreases with increasing depth. Lastly, we discuss the role of niche partitioning in facilitating the existence of a taxonomically and ecologically diverse assemblage of cryptobenthic fishes at mesophotic depths. This study represents the first attempt to examine the evolution of multiple lineages of deep-reef fishes in any tropical region and may serve as a primer for future studies investigating the evolutionary origins of the deep-reef fauna in other taxa and regions of the world tropics.

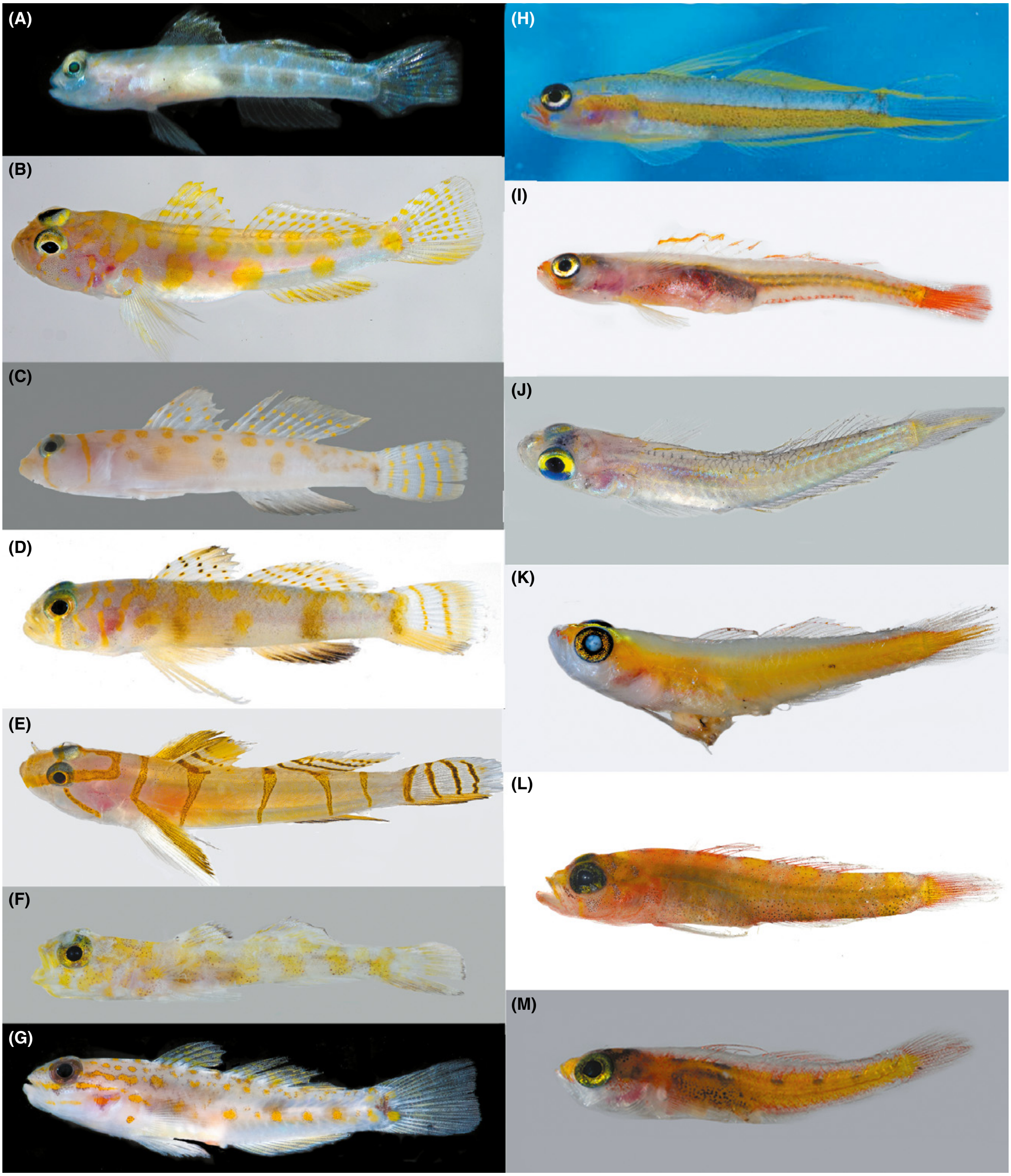

Fig. 1 Deep-reef Caribbean gobies included in this study. (A) *Pinnichthys aimoriensis* Espirito Santo, Brazil; (B) *Varicus cephalocellatus*, Bonaire; (C) *Varicus decorum*, Curaçao; (D) *Varicus veliguttatus*, Curaçao; (E) *Psilotris laurae*, Bonaire; (F) *Varicus* sp. 1, Bonaire; (G) *Coryphopterus curasub*, Curaçao; (H) *Antilligobius nikkiae*, Curaçao; (I) *Palatogobius* sp. 1, Curaçao; (J) *Palatogobius grandoculus*, Curaçao; (K) Gobiidae sp. 1, Bonaire; (L) Gobiidae sp. 2, Curaçao; (M) Gobiidae sp. 3, Curaçao. Photographs by Hudson Pinheiro, Carole Baldwin, Ross Robertson and Pat Colin.

Table 1 Deep-reef goby species included in this study

Species	Recorded depths (m)	Method of capture	Clade	Habitat use
Pinnichthys aimoriensis	70	Rebreathers	*Nes* subgroup	Benthic, solitary
Psilotris laurae	113–137	*Curasub*	*Nes* subgroup	Benthic, solitary
Varicus cephalocellatus	114–159	*Curasub*	*Nes* subgroup	Benthic, solitary
Varicus decorum	99–251	*Curasub*	*Nes* subgroup	Benthic, solitary
Varicus veliguttatus	153–287	*Curasub*	*Nes* subgroup	Benthic, solitary
Varicus sp. 1	250	*Curasub*	*Nes* subgroup	Benthic, solitary
Antilligobius nikkiae	90–198	*Curasub*	*Microgobius* group	Hovering, schooling
Palatogobius grandoculus	224–276	*Curasub*	*Microgobius* group	Hovering, schooling
Palatogobius sp. 1	117–128	*Curasub*	*Microgobius* group	Hovering, schooling
Coryphopterus curasub	70–80	*Curasub*	*Coryphopterus*	Benthic, solitary
Gobiidae sp. 1	120–140	*Curasub*	*Priolepis* lineage	Hovering, schooling
Gobiidae sp. 2	248	*Curasub*	*Priolepis* lineage	Unknown
Gobiidae sp. 3	128–161	*Curasub*	*Priolepis* lineage	Hovering, schooling

Methods

Specimen collection and phylogenetic analysis

Collection methods for most of the deep-reef species in this study follow that of Baldwin & Robertson (2014). Specifically, 12 of the 13 deep-reef species used in this study were captured by the *Curasub* submersible during several dives from 2011 to 2014 (Fig. 1B–L) in Curaçao and Bonaire. Each of the more than 130 Curasub dives between 2011 and 2015 typically involved 3–6 h of time spent searching for fish below 60 m. During dives, the fish anaesthetic quinaldine was pumped from a reservoir to a tube connected to one hydraulic arm, and a suction hose on the sub's other hydraulic arm collected fishes and deposited them into an acrylic container outside the submersible. Specimens were then retrieved at the surface where they were photographed and tissue sampled. Tissues were stored in saturated salt–DMSO (dimethyl sulphoxide) buffer. In addition, one other deep-reef species (*Pinnichthys aimoriensis*) was collected by Hudson Pinheiro and Thiony Simon off Espirito Santo, Brazil, using closed-circuit rebreathers (Fig. 1A). Whole genomic DNA was extracted from tissue samples using an automated phenol:chloroform extraction protocol on the Autogenprep965 (Autogen, Holliston, MA, USA). Four genes were sequenced for phylogenetic analysis: mitochondrial cytochrome *b* (cytb); the nuclear gene G protein-coupled receptor (GPR85, aka SREB2); the nuclear gene recombination activating gene subunit 1 (RAG1); and the nuclear gene zinc finger 1 protein (zic1). We did not use the ribosomal regions from Agorreta *et al.* (2013) due to difficulty with amplification and questions regarding the homology of poorly aligned regions. Primers for PCRs and thermal profiles are identical to those used in Agorreta *et al.* (2013).

New sequences from this study were combined with a reduced data set from Agorreta *et al.* (2013), in which we removed three problematic or 'rogue' taxa (*Kraemeria cunicularia*, *Schlindleria praematura*, *Schindleria pietschmanni*) whose phylogenetic position could not be resolved and inclusion in the analysis significantly reduced posterior probabilities across the entire tree. To better capture the diversity of Caribbean reef gobies, this alignment was then supplemented with cytb and RAG1 sequences from several species from the genus *Lythrypnus*, a shallow-reef group from the western Atlantic and eastern Pacific Oceans (sequenced by Maxfield *et al.* 2012). We also included sequences for the shallow-water members of the genus *Coryphopterus* (12 of 13 species, 92%), several additional shallow-water species of Gobiosomatini and the Butidae species *Kribia nana* to assist with molecular clock calibration (details below). Sequences were assembled and aligned in Geneious R6 (Biomatters Ltd., available at http://www.geneious.com). The total alignment had a length of 4395 bp (83% complete) and included 257 taxa, 35 more taxa than the largest gobioid phylogeny to date (Agorreta *et al.* 2013). Of particular importance, our data set is the most comprehensive in terms of western Atlantic goby species: our matrix includes 50 of the 55 genera of Caribbean gobies (91%), including 27 of the 30 genera of the Gobiosomatini (92%), and 23 of the 25 genera of non-Gobiosomatini gobies (90%). Partitioning scheme and substitution model choice were chosen with PARTITIONFINDER version 1.0.1 (Lanfear *et al.* 2012) using the 'greedy' algorithm and the Bayesian information criterion. The final alignment is available on Dryad (doi:10.5061/dryad.c3k7s).

The concatenated partitioned data set was analysed using Bayesian phylogenetic inference with MRBAYES version 3.2.1 (Ronquist *et al.* 2012) on the CIPRES portal version 3.3 (Miller *et al.* 2010). The analysis was replicated 4 times, each replicate consisting of two parallel MCMC runs (four chains each) for a length of 20 million generations. Convergence of parallel runs was assessed by comparing plots of ln L scores vs. generation in TRACER ver.1.5 (Rambaut & Drummond 2007), the standard deviation of split frequency statistic and the Potential Scale Reduction Factor statistic from MRBAYES, and by visually inspecting the resulting topologies of maximum-clade credibility (MCC) trees from each run. We assessed adequate mixing of MCMC chains by examining estimated sample sizes (ESS) from each run in TRACER.

Divergence-time estimation and ancestral habitat inference

We used an uncorrelated, lognormal, relaxed-clock model to infer divergence times using the program BEAST 2.2.3 (Bouckaert *et al.* 2014), as our data did not fit a strict molecular clock (likelihood ratio test, chi-square distribution, $P > 0.05$). We used the following three calibration points. First, following Thacker (2014), we set the crown age of all gobioid fishes (root age of the tree) to a minimum of 52 Myr with a very soft upper bound (exponential distribution, mean = 10, offset = 52), based on the oldest known fossil gobioid, an Eocene otolith (Bajpai & Kapur 2004; Gierl *et al.* 2013). The most recent common ancestor of *K. nana* and *Bostrychus zonatus* was set to a minimum of 23 Myr with a soft upper maximum (lognormal distribution, mean = 1.5, sigma = 0.8, offset = 23), based on a well-preserved whole body fossil of an upper Oligocene Butidae species that was determined to be the sister taxa of *Kribia* (Gierl *et al.* 2013). Lastly, the most recent common ancestor of the Atlantic and Pacific species of *Gobulus* was set to a minimum age of 3 Myr with a soft upper maximum (gamma distribution, $\alpha = 1.9$, $\beta = 3.0$, offset = 2.5), based on the final closure of the Isthmus of Panama, which marks the latest point in which this split could have occurred. While the date of the final closure of the Isthmus of Panama is still contentious (e.g. Bacon *et al.* 2015a,b; Lessios 2015; Marko *et al.* 2015; Montes *et al.* 2015), the combination of a hard minimum and soft upper maximum in our calibration point accommodates ages from multiple hypotheses and allows for flexibility in the inference of the split between *Gobulus* species. In addition, despite uncertainty in the identification of gobioid otoliths, we chose to use the *c.* 52-million-year-old Eocene otolith fossil of Bajpai and Kapur (2004) for the root gobioid calibration point rather than the oldest articulated skeleton (*c.* 44 Ma; Gaudant 1996), as this older fossil agrees better with the considerably older age estimates of the origin of gobioids derived from other phylogenetic analyses that included wider sampling, albeit without fossil gobioid calibration points (Near *et al.* 2012; Betancur-R *et al.* 2013). To remedy the problem of the BEAST analysis not converging, we used a fixed topology for the analysis. To do this, we converted our MCC tree from MRBAYES to an ultrametric tree using the penalized likelihood approach (Sanderson 2002) with the above calibration points, and inputted this as our starting tree, fixing topology by disabling topology-sampling MCMC operators in the BEAST analysis. For the BEAST analysis, we partitioned our data set by gene and assigned each partition a HKY + G substitution model, assigned different clock models for the cyt b and the group of three nuclear genes, and implemented a Yule speciation prior with parameters conditional on the root age of the tree. The analyses were run four times, each with 30 million generations. We compared plots of posterior probability vs. generations for each analysis in TRACER, ensured adequate ESS (>200), and visually inspected divergence times of the four MCC trees to see that each analysis recovered similar results.

To infer the frequency, dates and topological location of habitat transitions between shallow-water and deep reefs on our phylogeny, we used stochastic character mapping of discrete traits via SIMMAP (Bollback 2006) in R (R Core Team 2015), through the function *make.simmap* ('PHYTOOLS' package; Revell 2012). Species in our phylogeny were coded as being deep-reef species if they are known exclusively from depths at or below 60 m. Species with depth ranges that extend no deeper than 60 m were coded as shallow-water species. Very few species of Caribbean reef gobies (four species of *Coryphopterus*, four species of *Elacatinus* and *Priolepis hipoliti*) that are primarily shallow-reef species have been recorded (rarely) in upper-mesophotic depths. More than 130 *Curasub* dives of 3- to 6-h duration/dive spanning 5 years have yielded only one specimen of *P. hipoliti* from 107 m and only three *Coryphopterus venezuelae* from 65 to 69 m. None of the other 49 species of shallow-reef gobies from Curaçao were observed or collected below ~60 m. Lastly, species in the genus *Bollmannia* frequently extend into mesophotic depths, but these species are not reef specialists and instead occur in association with burrows over flat mud bottoms that are largely homogeneous with respect to depth. Because we are primarily interested in transitions into deep-reef habitats that are ecologically distinct from shallow-water habitats, we coded *Bollmannia* as shallow-water species for the purpose of the SIMMAP analysis.

The SIMMAP analysis assumed a Markov model with a symmetrical rate matrix for character-state changes, where changes between-character states in either direction were considered equally probable a priori. We ran the SIMMAP analysis on both a single MCC tree from our BEAST analysis, as well as a subset of 100 random trees from the post-burn-in posterior distribution of trees from the BEAST analysis. The subset of post-burn-in trees was analysed to accommodate divergence-time uncertainty in our estimation of the timing of habitat transitions. For the analysis of the single MCC tree, we used 10 000 MCMC iterations to sample the posterior distribution of possible shallow/deep transition rates. For computational efficiency, in the analysis of 100 post-burn-in trees, we used a single maximum-likelihood point estimate for estimating the transition rate matrix rather than using MCMC. Each SIMMAP analysis included 100 character-mapping simulations per tree (100 trees × 100 simulations = 10 000 total simulations for our analysis of 100 post-burn-in trees). For the SIMMAP analysis of our single MCC BEAST tree, the results of all simulations were visually summarized on the tree using the function *density.map* ('PHYTOOLS' package; Revell 2012). We also extracted the estimated dates of habitat transitions for each unique lineage of deep-reef gobies from our SIMMAP analysis of post-burn-in trees. These estimates of transition times were plotted as histograms, and we compared the timing of habitat transitions across groups using ANOVA to determine whether independent groups of gobies were simultaneously transitioning between shallow and deep habitats.

Depth distributions of Caribbean reef gobies

Rather than sampling in both shallow and deep water that would enable the identification of any clear-cut break point between euphotic (shallow) and mesophotic (deep) faunas, studies of mesophotic fishes typically involve sampling only below some arbitrary depth between ~30 and 60 m. Hence, to provide a less subjective estimate of the depth zone at which there might be a changeover between the euphotic and mesophotic components of the Greater Caribbean reef-goby fauna, we examined the known depth distributions of all members of that fauna. Using those data, we assessed whether and at which depth there is any peak in the relative abundance of depth-range endpoints (both depth maxima and minima) that would indicate a changeover in faunal composition (see Roy *et al.* 1998).

To determine depth distributions of gobies, we collected records for the 95 species that are associated with reefs in the western Atlantic. To mitigate biases from incomplete data, we limited our depth data to species from the western Atlantic Ocean where our groups of interest occur, and where the information on goby depth distributions and diversity is considerably more complete. Unlike most western Atlantic gobies, for which collections are abundant and identifications of species have been verified by the authors, a great number of Indo-Pacific gobiid taxa are poorly known and virtually no depth-range data are available—especially for deep-reef taxa. Depth data used here were gathered from databases based only on physical collections rather than visual observations, given the inherent difficulty of identifying many species of gobies in the field. Specifically, we used data from Robertson & Van Tassell (2015), supplemented with museum records from the American Museum of Natural History online database and from FISHNET2 (www.fishnet2.com), which queries 73 natural history collections from around the world. To highlight bathymetric breaks, we divided the euphotic–mesophotic zone into 10-m intervals and calculated the total number of species that were present in each depth interval. We then refined this count by tabulating the number of species with depth minima or maxima in each interval. Lastly, to remove the effect of uneven species richness across depth intervals and identify potential bathymetric faunal breaks, we divided the total number of species with depth endpoints (minimum or maximum) in each interval by the total number of species present in that interval, giving us a measure of the relative number of species that start or end in the given 10-m depth interval. For species present at 250–300 m, we refrained from designating a specific depth maximum due to infrequent collections and the sampling bias associated with the maximum depth reachable by the *Curasub* (300 m).

Results

Phylogenetic analysis

Thirteen deep-reef species were recovered in three monophyletic clades and two single-species lineages within the Gobiidae (Fig. 2). The majority of the deep-reef species belong to the tribe Gobiosomatini. Within that tribe, three species in two genera, *Antilligobius* and *Palatogobius*, were recovered within the *Microgobius* group (*sensu* Birdsong *et al.* 1988; Van Tassell *et al.* 2012), and the two genera were recovered as sister taxa with strong support. The remaining six deep-reef species of gobiosomatins belong to the *Nes* subgroup (*sensu* Van Tassell *et al.* 2012; Tornabene *et al.* 2016), including four species of *Varicus* (*V. cephalocellatus*, *V. decorum*, *V. veliguttatus*, and the undescribed *Varicus* sp. 1; Fig. 1B–D,F), one *Psilotris* (*P. laurae*; Fig. 1E) and one *Pinnichthys* (*P. aimoriensis*; Fig. 1A). The mesophotic species *Coryphopterus curasub* was recovered as sister to a clade containing *Coryphopterus dicrus*, *Coryphopterus glaucofraenum*, *Coryphopterus tortugae* and *Coryphopterus venezuelae*. *Coryphopterus* was recovered as sister to

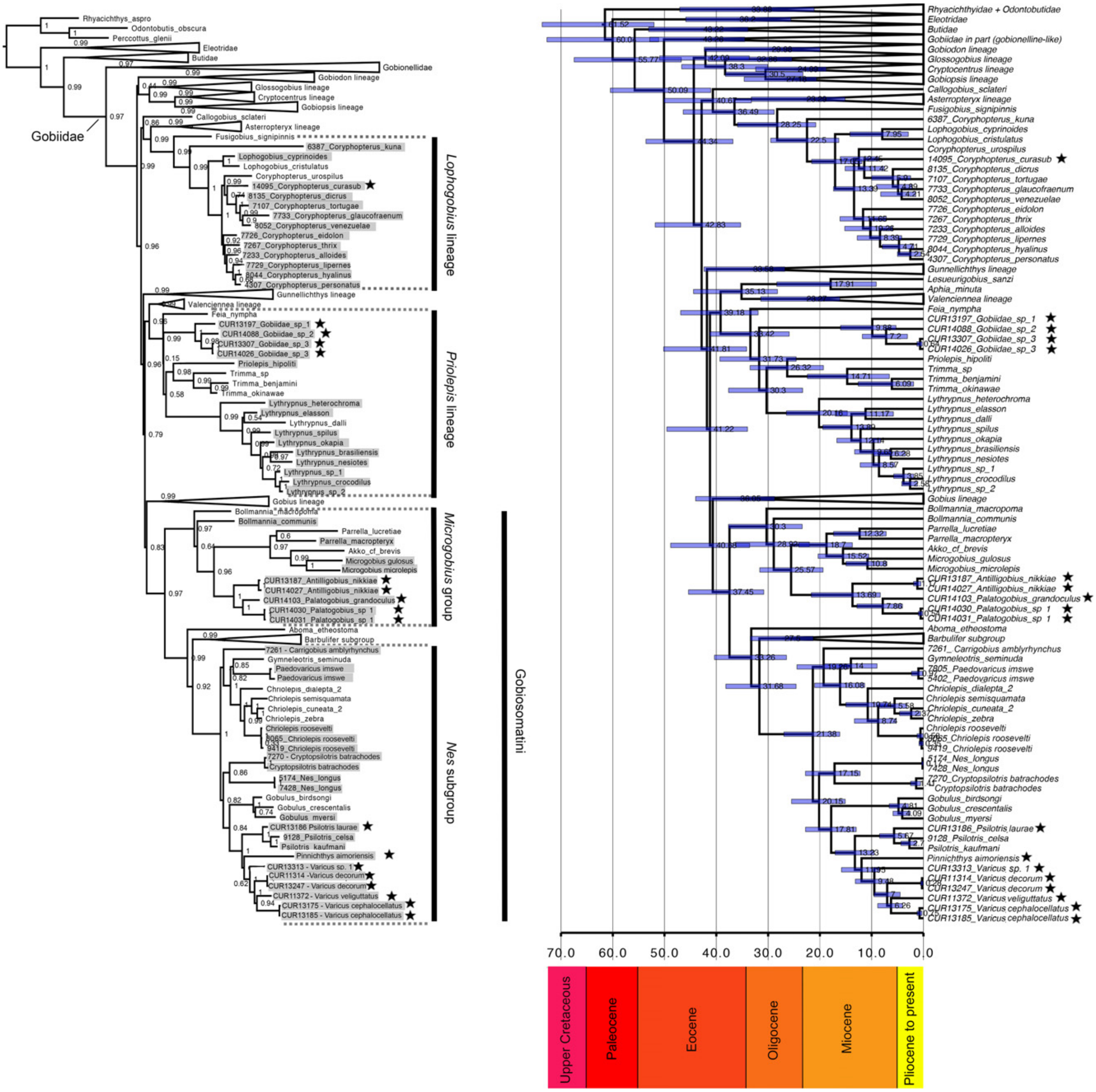

Fig. 2 Bayesian maximum-clade credibility trees of selected Gobiidae. (A) Phylogram from MRBAYES, node labels are posterior probabilities. (B) Time-calibrated phylogeny from BEAST, node labels and bars are median divergence-time estimates with 95% highest posterior density intervals. Clade names follow that of Agorreta *et al.* (2013) and Van Tassell *et al.* (2012). Western Atlantic species are highlighted in grey. Stars denote deep-reef species.

Lophogobius, with the western Atlantic *Coryphopterus kuna* falling outside those taxa. The remaining three deep-reef species (Gobiidae sp. 1, sp. 2 and sp. 3; Fig. 1K–M) represent a new genus and form a single clade within the *Priolepis* lineage (*sensu* Agorreta *et al.* 2013), which also contains the New World genus *Lythrypnus*, the Indo-Pacific genera *Trimma* and *Feia*, and the Indo-Pacific/Atlantic genus *Priolepis* (Fig. 2).

Divergence-time estimation and mapping of habitat transitions

Our SIMMAP analysis (Fig. 3) indicates that there were between 4 and 6 (mean 5.2) transitions between shallow-water and deep-reef habitats in our study group. The number of habitat transitions on the tree differed depending on the likelihood estimation of the

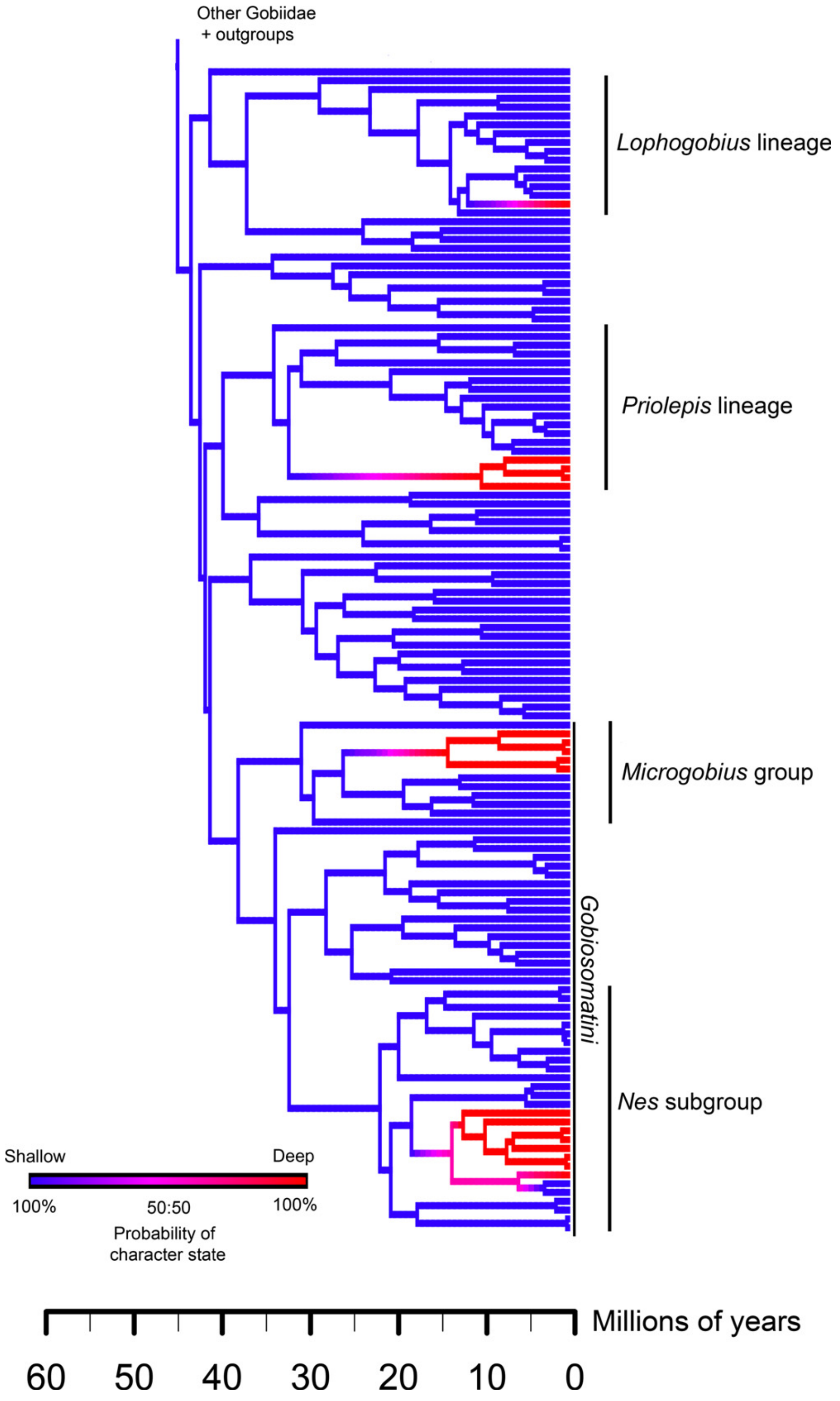

Fig. 3 Ancestral habitat reconstruction for Gobiidae (showing relevant subclade only) from 1000 stochastic-mapping simulations on maximum-clade credibility tree from BEAST analysis. Red branches have 100% posterior probability of occupying deep-reef habitat; blue branches have 100% posterior probability of occupying shallow-water habitat.

transition rates and the branch lengths of individual tree analysed. Most habitat transitions were from shallow water to mesophotic habitats (mean 4.49 transitions per simulation), while deep-to-shallow habitat transitions were far less common (mean 0.73 transitions per simulation). The only potential case of deep-to-shallow habitat transition occurred within the *Nes* subgroup, as a reversal within an otherwise deep-water clade. In this group, the SIMMAP analysis inferred two different scenarios for the evolution of deep-reef species (Figs 3 and 6); (i) a shallow-to-deep transition occurred on the branch leading to the clade containing *Varicus*, *Psilotris* and *Pinnichthys*, followed by a deep-to-shallow reversal in the ancestor of *Psilotris celsa* and *Psilotris kaufmani*; or (ii) two shallow-to-deep transitions, one in the ancestor of the *Varicus* + *Pinnichthys* clade and the other in *P. laurae*. The latter scenario is more probable as it occurred in approximately twice the number of simulations as the former scenario (Fig. 4).

From a familywide perspective, our SIMMAP analysis indicates that shallow-to-deep transitions occurred primarily during two distinct phases (Fig. 4). The first phase occurred between 10 and 30 Ma and involved two lineages, the *Antilligobius* + *Palatogobius* clade and a clade of three undescribed species in the *Priolepis* lineage. The second phase, which involved at least the divergence of *Coryphopterus curasub* from the shallow members of *Coryphopterus*, occurred within the last 10 Myr. Depending on the SIMMAP scenario, members of the *Nes* subgroup may have transitioned to deep reefs during both the early and late phases (if *P. laurae* transitioned to deep reefs independently of *Varicus* + *Pinnichthys*), or exclusively during the early phase (if the transition occurred on the branch leading to *Varicus* + *Pinnichthys* + *Psilotris*). In the latter scenario, the timing of the single deep-to-shallow reversal in *Psilotris* is consistently estimated within the last 10 Myr (mean 4.71 Ma). Although a familywide perspective indicates two major pulses of shallow-to-deep transitions over time, as evident by the strongly bimodal distribution in Fig. 4, the mean estimates for the dates of habitat transitions from a clade-by-clade perspective are significantly different from one another (ANOVA, $P < 0.001$; pairwise Tukey's HSD $P < 0.001$; Figs 5 and 6), indicating that each transition could arguably be considered temporally distinct.

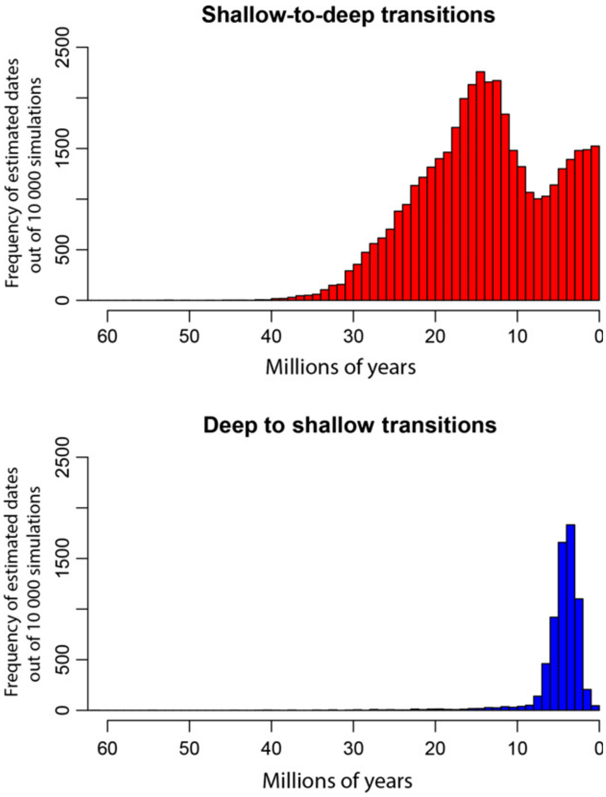

Fig. 4 Age estimates of habitat transitions across Greater Caribbean members of the family Gobiidae from SIMMAP simulations on 100 trees from BEAST analysis.

Finally, our results indicate three instances in which evolutionary lineages have subsequently diversified after transitioning to deep reefs (*Nes* subgroup, *Priolepis* lineage, *Antilligobius* + *Palatogobius* lineage). Only in the most recent shallow-to-deep transitions (*Coryphopterus curasub* and perhaps *P. laurae*) do deep-reef species appear to represent single evolutionary offshoots. The one possible deep-to-shallow transition led to subsequent diversification of shallow-water *Psilotris* (*P. kaufmani* and *P. celsa*).

Depth distributions of Caribbean reef gobies

Depth distribution data for our 13 species of deep-reef gobies indicate very little depth range overlap between deep and shallow members of the *Nes* group, *Microgobius* group and *Priolepis* lineage (Fig. 7). Data for Caribbean reef gobies as a whole indicate that species richness decreases as depth increases (Fig. 8A). Most species (67) have the upper limit of their depth distributions between 0 and 10 m, and very few species have the lower limit of their depth distributions below 70 m (Fig. 8B). The depth intervals that contain the most species' depth maxima are 21–30 m (15 species) and 51–60 m (12 species; Fig. 8B). Two peaks in the relative abundance of depth-range endpoints are evident in Fig. 8C: at 0–10 m and between 51 and 70 m. Deeper peaks than the latter could well represent sampling artefacts due to small numbers of species present.

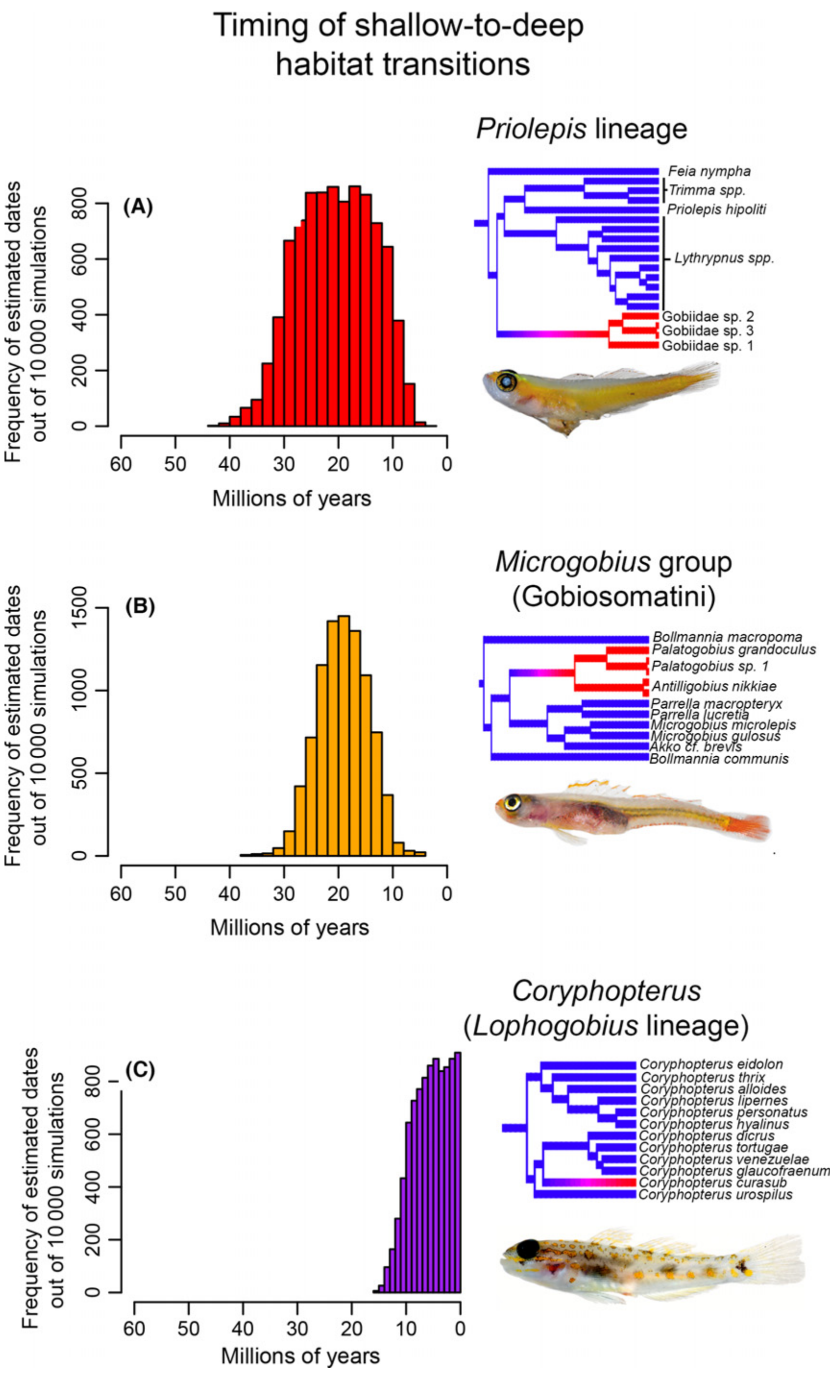

Fig. 5 Age estimates of habitat transitions for selected clades of Greater Caribbean gobies. (A) *Priolepis* lineage shallow-to-deep transition; (B) *Microgobius* group shallow-to-deep transition; (C) *Coryphopterus* (*Lophogobius* group) shallow-to-deep transition. Note differences in *Y*-axis scales.

Discussion

Eco-evolution of deep-reef species in the Microgobius group (Gobiosomatini)

Antilligobius and *Palatogobius*, sister taxa in our phylogeny (Fig. 2), share several ecological characteristics that are unique within the *Microgobius* group. In addition to occurring at greater depths, *Antilligobius* and *Palatogobius* are the only group members typically found hovering in the water column over hard substrates. In contrast, most species of other genera in that group (*Bollmannia*, *Akko*, *Microgobius* and *Parrella*) are strictly associated with soft mud and fine sediments, and many are known to use burrows as substrate refuges. *Antilligobius* and *Palatogobius* were commonly observed during *Curasub* dives hovering up to 50 cm above rocky-reef slopes or in front of small caves or

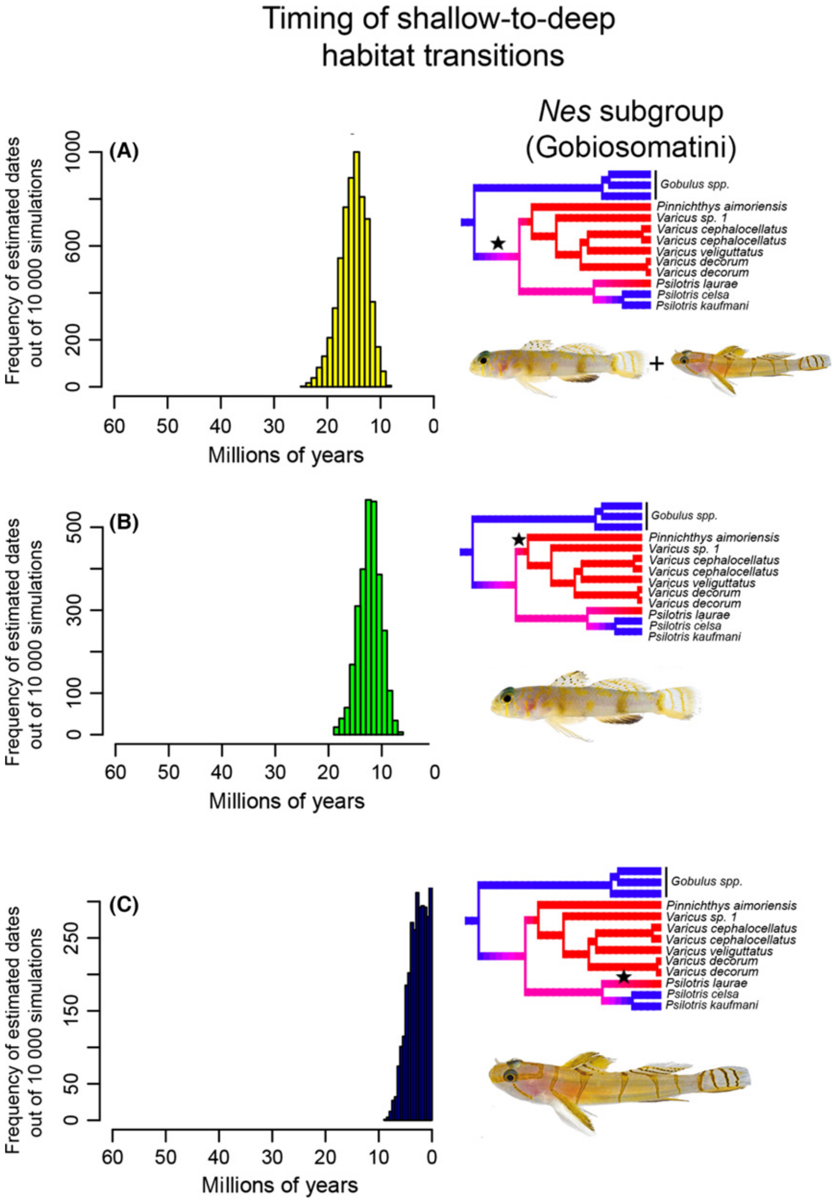

Fig. 6 Age estimates of habitat transitions for two possible evolutionary scenarios within the *Nes* subgroup. (A) Scenario wherein habitat transition occurred on branch leading to (*Varicus* + *Pinnichthys*) + *Psilotris* clade; (B) and (C). Scenarios wherein independent habitat transitions occurred in the *Varicus* + *Pinnichthys* clade (B) and in the *Psilotris* clade (C), respectively. Note differences in *Y*-axis scale in each case.

crevices along vertical rocky walls in Curaçao, often in groups of tens to scores of individuals, and did not retreat to benthic shelters when disturbed. Such near-bottom schooling behaviour is rare in the *Microgobius* group and in gobies in general. When hovering in schools, *Antilligobius* feeds on copepods and other zooplankton (F. Young & D. Marine, personal communication). Similarly, *Palatogobius* species may also be planktivorous. An increase in the percentage of planktivorous fishes with increasing depth along a reef slope has been documented in the Indo-Pacific (Thresher & Colin 1986; Brokovich *et al.* 2008), and schooling coupled to planktivory may be selectively advantageous features for fish lineages expanding onto deep reefs. *Antilligobius* and *Palatogobius* display some morphological features that may be adaptive for a hovering lifestyle at mesophotic depths. Although the gas bladder is absent or variably developed in many gobiid groups (McCune & Carlson 2004), it is present in *Antilligobius* and *Palatogobius* sp. 1, and a modification of the haemal arch in these species (see Van Tassell *et al.* 2012: Fig. 8B) allows the well-developed gas bladder to extend throughout the elongate abdominal cavity and into the expanded haemal arch. The cleared and stained specimens identified as *Palatogobius paradoxus* examined by Van Tassell *et al.* (2012) had neither an expanded haemal arch nor a gas bladder. However, radiographs of the holotype of *P. paradoxus* and recently available photographs of what appears to be *P. paradoxus* or another species of *Palatogobius* from Curaçao

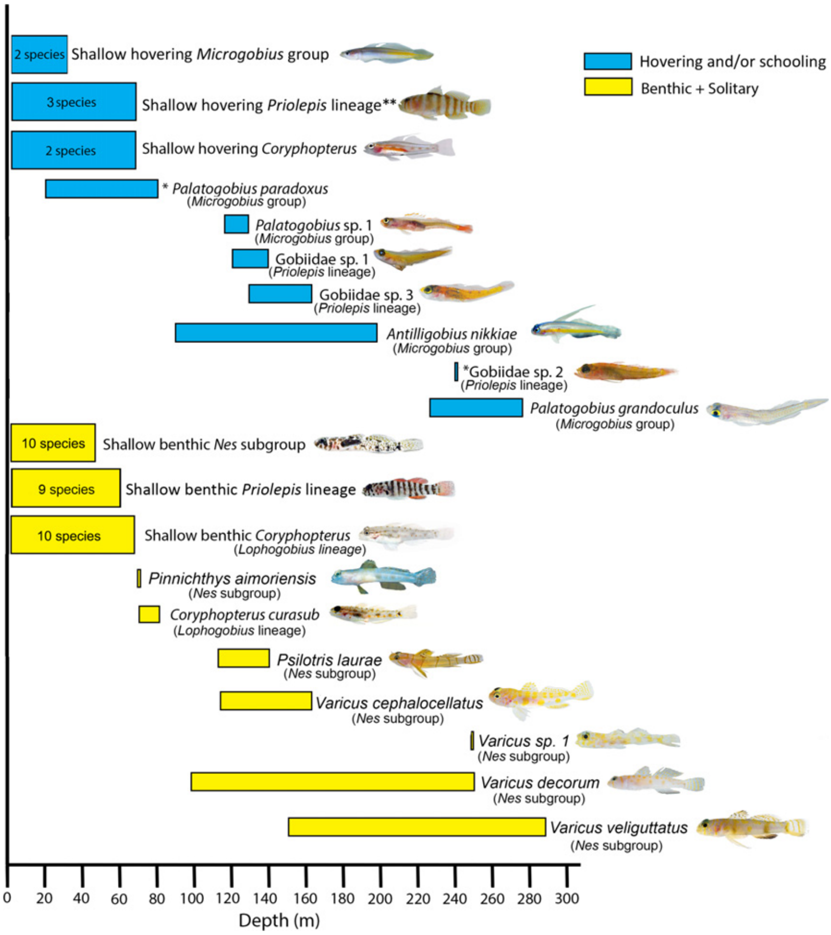

Fig. 7 Depth distributions for the two ecotypes of deep-reef Greater Caribbean gobies. *Uncertainty regarding a hovering and schooling vs. benthic and solitary lifestyle. **Only one specimen of 593 records of *Priolepis hipoliti* was collected below this range to 107 m.

do show an expanded haemal arch and well-developed gas bladder. Currently, *P. paradoxus* is known from shallower depths (20–80 m, Greenfield 2002) than either *Palatogobius grandoculus* or *Palatogobius* sp. 1 (see Table 1; Fig. 7). A well-developed gas bladder may provide an energy-efficient means of controlling buoyancy, particularly in the resource-limited environment of deep reefs (Kahng *et al.* 2011). Further, such a bladder may also serve to amplify sound for enhancing communication in low-light habitats. Sound production is an important method of communication in gobies, and several species are known to produce sounds for both sexual and aggressive signalling (e.g. Mok 1981; Tavolga *et al.* 1981; Torricelli *et al.* 1990; Lugli *et al.* 1995; Malavasi *et al.* 2008; Horvatić *et al.* 2015). An expanded haemal arch has evolved independently in several other groups of hovering gobiids, including the pantropical genus *Ptereleotris* (Birdsong *et al.* 1988) and some species of the Indo-central Pacific genus *Trimma* (Winterbottom 1984; Winterbottom *et al.* 2007), where it was hypothesized to serve a similar function with regards to allowing expansion of the gas bladder. Although some Indo-Pacific representatives of *Ptereleotris* and *Trimma* (an exclusively Indo-Pacific genus) have been observed at mesophotic depths and form small groups or schools, and those species were not available for inclusion in our phylogeny.

Our SIMMAP analysis on the sample of post-burn-in BEAST trees indicates that the transition from shallow water to deep reefs in the *Antilligobius* + *Palatogobius* clade occurred *c.* 10–30 Ma (mean 19.2 Ma; Fig. 5B). This overlaps broadly with the deep-reef transition in a clade of three new species from the *Priolepis* lineage (Fig. 5A), as well as with the earliest estimates of the deep-reef transition in the *Nes* subgroup (Fig. 6A). The three species in the *Priolepis* group also display hovering behaviour, and at least one of them (Gobiidae sp. 3) has been observed in schools of hundreds of individuals at Curaçao. In one instance, the *Curasub* collected both *Palatogobius* sp. 1 and Gobiidae sp. 3 from a single mixed school of scores of fishes that included individuals of both species.

The *Antilligobius* + *Palatogobius* clade represents a clear example of a lineage diversifying on deep reefs. After the initial shallow-to-deep transition at the base of this clade, the split between *Antilligobius* and

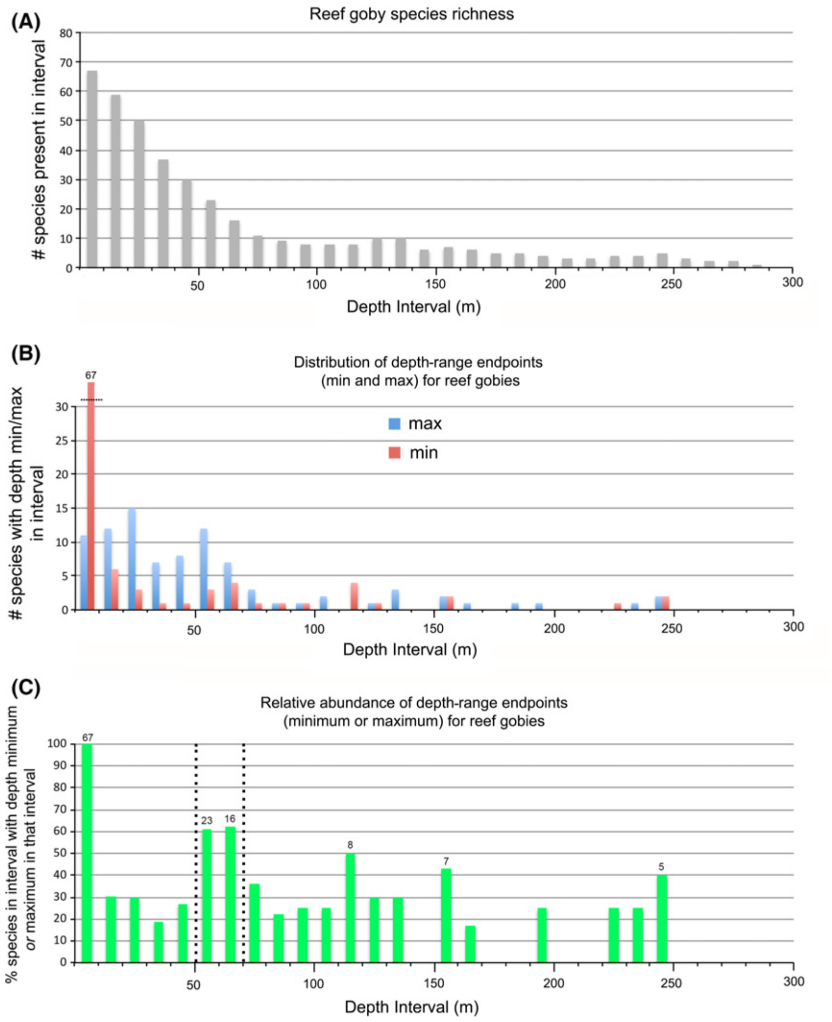

Fig. 8 Depth distribution data for 95 species of Greater Caribbean reef gobies. (A) Total number of species present in each depth interval; (B) total number of species that have their maximum or minimum depth within each 10-m interval; (C) per cent of species present in a 10-m depth interval that have their minimum or maximum depth within that interval. Dotted lines indicate the inferred transitional zone between the shallow-reef and deep-reef faunas. Numbers above select bars indicate the total number of species present in that interval.

Palatogobius was estimated at 13.7 Ma [95% highest posterior density (HPD) = 8.3–21.2 Ma], followed by the divergence between the two *Palatogobius* species *c.* 7.9 Ma (95% HPD = 4.1–12.8 Ma). In addition to these two speciation events, Gilbert (1971, 1977) noted that there may be an additional undescribed species of deep-water *Palatogobius* from Panama, indicating another possible speciation event at depth. With the exception of the *Curasub* specimens, the few specimens of *Palatogobius* in collections have come from trawls or dredges primarily over flat bottoms. In contrast, observations from *Curasub* dives indicate that some species in this genus are abundant and conspicuous at the junction of sand and rock substrata with rocky reefs. The exception is *P. paradoxus*, which is known from shallower water and lives on unconsolidated bottoms. If the deep-living species in this genus indeed prefer more complex substrates that are intentionally avoided by benthic sampling gear, the genus may contain many more species than those currently known simply due to a sampling bias. In contrast, *Antilligobius nikkiae* has been observed or collected from Belize, Bahamas, Curaçao, Cuba, Mexico and Puerto Rico, and all morphological evidence suggests the presence of a single species throughout the Caribbean. However, this has yet to be tested with DNA sequence data. Nonetheless, the *Antilligobius* + *Palatogobius* clade as a whole presents another example of a lineage of Gobiosomatini that has invaded a novel niche and subsequently diversified, providing additional support that the Gobiosomatini is one of the best known cases of adaptive radiation in the marine environment (Rüber *et al.* 2003).

Eco-evolution of deep-reef fishes within the Nes subgroup (Gobiosomatini)

The SIMMAP analysis suggests that in the *Nes* clade (Fig. 2) there was either a single shallow-to-deep transition followed by a deep-to-shallow transition in *Psilotris* (Fig. 6A) or that there were two independent shallow-to-deep transitions (Fig. 6B,C). The latter scenario appeared in approximately twice the number of SIMMAP simulations as the former, and hence was statistically more probable. Pending additional investigation, we consider both scenarios equally plausible at this point.

With the exception of a single species, *Nes longus*, which typically lives in burrows with *Alpheus* snapping shrimp, shallow-water species in this group are considered to be strictly cryptobenthic, occurring around various rock or calcareous substrates in the littoral or sublittoral zone. Many species occupy tiny interstitial spaces on or around coral and rocky reefs. This habitat specialization may have coincided with several morphological adaptations, including a reduction in body size (standard length < 20 mm in many species), loss of pelvic fin fusion, reduction in sensory head pores and canals, reduction in squamation and cryptic coloration (Findley 1983). The deep-reef members of the *Nes* clade have retained many of these characters, but differ ecologically in some regard. Most deep-reef species in the *Nes* subgroup are larger and more robust than their shallow-water relatives and have bright yellow body coloration; several were collected while perching on open sand and rubble substrates. The phenotypic and behavioural differences between shallow and deep-reef species could well reflect different selective forces between the two habitats. Total fish abundance and diversity generally are lower on deep reefs in comparison with shallow reefs (Brokovich *et al.* 2008; Rosa *et al.* 2015), which may result in fewer predators (both in abundance and diversity) and less competition for space and resources. A greater availability of niche options in deep-reef habitats than shallow habitats, where goby diversity and abundance is much higher (e.g. Brokovich *et al.* 2008), may explain the strong bias towards shallow-to-deep transitions over deep-to-shallow transitions (4.4 vs. 0.7) among Caribbean gobies. Factors such as reduced competition and predation on deeper reefs could relax the evolutionary constraints on body size and reduce the selective advantages of an extreme cryptobenthic lifestyle. In this regard, the invasion of deep reefs by *Nes*-clade gobies from shallow-water habitats may have created a speciation opportunity by enabling the use of deep-reef niches unoccupied by other goby species. This 'ecological release' may have enabled the subsequent speciation events in the deep-reef lineages of this clade, facilitating a radiation into an otherwise species-depauperate ecosystem from the mid-Miocene and throughout the Pliocene. There may be additional speciation events at depth in the *Nes* subgroup not reported here. Several deep-water species in the *Nes* clade were not sampled in our study (e.g. *Pinnichthys prolata*, *P. bilix*, *P. atrimela*, *Varicus benthonis*, *V. vespa*, *V. bucca*, *V. marilynae*), although recent combined molecular and morphological analyses strongly support that these species are members of the deep-reef clades identified here (Tornabene *et al.* 2016). Thus, it is probably that the deep-reef radiation within the *Nes* subgroup is even more substantial than our phylogeny depicts.

Eco-evolution of deep-reef fishes in the Priolepis and Lophogobius lineages

Three deep-reef species (Gobiidae sp. 1–3 in Fig. 2) were recovered in the *Priolepis* lineage—an exceptionally diverse clade consisting primarily of species that live on shallow reefs (Fig. 2). There are scant records of gobies from this group in the Indo-Pacific region from depths that extend marginally, or in some cases considerably, into the mesophotic zone. None of these Indo-Pacific species were available for inclusion in this study, as very few gobies have been collected from tropical Indo-Pacific deep reefs. The mean estimate for the timing of the shallow-to-deep habitat transition in the *Priolepis* lineage is 20.4 Ma (standard deviation = 7.0 Myr; Fig. 5A). However, the age of this lineage is probably to change with increased taxon sampling. We are relatively confident that the putative new genus (Gobiidae sp. 1–3) belongs in the *Priolepis* lineage (posterior probability = 1.0), but the position within this group is uncertain and may be clarified with the addition of more taxa. Our phylogenetic sampling of Indo-Pacific gobies is limited, and it is probably that the true sister group to the new genus is missing from our study. Increased sampling leading to the inclusion of a more closely related sister group to this clade would have two impacts on the inferred age of the shallow-to-deep transition: (i) the age of the new genus, and thus the age of the habitat transition, will be shifted towards the present, as the addition of a sister group will shorten the internal branch leading to the most recent common ancestor of Gobiidae sp. 1, sp. 2 and sp. 3; (ii) adding more taxa to the *Priolepis* group may cause the width of our age estimates to become narrower for this group (as well as for the corresponding habitat transition), due to additional information being available on mutations occurring along internal branches within the *Priolepis* lineage. These branches are currently very long and have ages that are difficult to estimate precisely, as indicated by the wide age range in Fig. 5A. Currently, the age estimates for the habitat transition in the *Priolepis* group overlap broadly with that of the *Antilligobius* + *Palatogobius* clade (Fig. 5B), and this overlap could become more precise with the addition of closely related species within the *Priolepis* lineage. Little is known about the ecology of Gobiidae sp. 1 and sp. 2, as both are known from only a limited number of specimens; however, Gobiidae sp. 3 is ecologically similar to

Antilligobius and *Palatogobius* in hovering above the substrate in small schools.

Coryphopterus curasub is the only known deep-reef member of the *Lophogobius* lineage and is one of the most recent invasions of deep reefs among the Greater Caribbean gobies studied here. The mean estimate for the timing of the shallow-to-deep transition for *C. curasub* is 5.49 Ma (standard deviation = 3.42 Myr; Fig. 5C). Species of *Coryphopterus* can be divided into two general ecological groups based on their behaviour. *Coryphopterus personatus* and *Coryphopterus hyalinus* are the smallest species in the genus, and both form large aggregations that hover in the water column within ~50 cm above the substrate where they feed on zooplankton. They are frequently the most numerically abundant species of gobies on shallow reefs in the Caribbean (Greenfield & Johnson 1999; Dominici-Arosemena & Wolff 2005). The remaining species in the genus, including the deep-reef *C. curasub*, are benthic species that typically perch on sand, rock or coral substrates, solitarily or in loose aggregations of a few individuals, and move about in contact with the substrate.

It seems paradoxical that planktivory, hovering and schooling are all common and potentially adaptive behaviours in deep-reef gobies, including several of those studied here, yet the species of *Coryphopterus* that display these behaviour are not found below 50–72 m depth, and the only 'true' mesophotic *Coryphopterus* collected to date is a demersal species found on sand and rubble (Baldwin & Robertson 2015; Fig. 6). This paradox may be related to the timing of diversification events and the sequence of habitat transitions across gobies as a whole, coupled with the temporal availability of niches. The mean age of the genus *Coryphopterus* is estimated at 13.36 Ma, and the origin of the clade containing the two hovering species is estimated at 2.54 Ma. Our analysis of the timing of habitat transitions indicates that by the middle-to-late Miocene (10–15 Ma), deep reefs were already inhabited by the three hovering planktivorous species from the *Microgobius* group, plus another three from the *Priolepis* lineage. Ecologically, these mesophotic species and the shallow hovering species of *Coryphopterus* are very similar, and could well occupy similar microhabitat and trophic niches on reefs. The presence of large hovering groups of *Antilligobius*, *Palatogobius* and Gobiidae sp. 3 on deep reefs could have restricted the ecologically similar hovering *Coryphopterus* species to shallow depths. Both the absence of hovering *Coryphopterus* on deep reefs and the restriction of *C. curasub* to the upper edge of the mesophotic zone may also be due, in part, to these relatively young species not having sufficient time to develop a complex set of habitat-specific adaptations for exploiting the mesophotic zone on Caribbean reefs.

Bathymetric trends and oceanographic considerations

Our depth data for all Caribbean gobies highlight two major trends. First, as observed in reef-building corals and reef fishes in general, goby species richness decreases as depth increases (Fig. 8A). The most prominent bathymetric break in Caribbean reef-goby distributions occurs between 50 and 70 m (Figs 7 and 8). Among them, 14 species (61%) that occur between 51 and 60 m and 10 species (63%) that occur between 61 and 70 m have depth ranges that either start or end in this zone (Fig. 8B,C). These high frequencies of depth minima and maxima in the 50–70 m zone suggest that it represents the transitional zone between the shallow-reef community and the true deep-reef fauna, at least in the case of this taxon and biogeographic region.

The exact depths of this transitional zone and of species depth ranges in general should be interpreted cautiously. The 50–70 m transitional zone may also be somewhat biased by historical collection patterns. The recreational scuba limit is ~40 m, and while there is a history of a handful of ichthyologists diving slightly below this limit to collect and observe fishes in the Caribbean, it is probably that this limit explains the known depth maxima for some species. However, in over 130 submersible dives spanning 5 years at Curaçao, there have been no observations or collections of shallow-water gobies at depths greater than 70 m, despite their abundance and diversity (49 species) on shallow reefs there. Thus, historical sampling bias alone cannot account for the 50–70 m transitional zone observed here. Beyond any potential sampling artefacts, the maximum depth of a species is probably determined by a complex combination of factors such as temperature, light and substrate—all of which may not correlate with depth in a uniform manner across a species' entire geographic range. Nonetheless, the preliminary bathymetric patterns shown here (Figs 7 and 8) reinforce conclusions of prior studies on reef fishes below 50 m, which, although limited in number and scope, collectively point to the existence of a unique, truly deep-reef fauna comprising species that are rare or absent from shallow reefs. Brokovich *et al.* (2008) noted that the bathymetric fauna breaks in reef fishes correlated with a decrease in branching corals with increasing depth. The overall drop in coral cover may explain why some species of gobies that are dependent on reef-building corals as adults are absent from mesophotic reefs (e.g. some *Elacatinus* spp., *Coryphopterus lipernes*). On the other hand, Srinivasan (2003) found that for several species of coral-reef fishes, there were significant differences in recruitment at different depths despite similar substrates across depths. This suggests that species depth preferences may be driven in part by other factors beyond substrate that could impact fishes at early life history stages, including abiotic factors such as light and temperature. We recorded water temperature for 1 year (September 2012 to August 2013) at 30-m increments along the deep-reef slope (0–250 m) at Substation Curaçao and found significantly different seasonal trends at euphotic vs. mesophotic depths. From September to December, depths 50 m and shallower routinely reached 29 °C, whereas depths from 75 to 250 m ranged from 15 to 25 °C. In late December, water temperatures at 50 m and shallower dropped sharply from 29 to 25 °C, whereas temperatures at deeper depths (180–250 m) remained constant or even increased by as much as 2 °C (75–220 m). While these data represent only a single year at a single locality, they illustrate the possibility that significant seasonal differences in temperature with depth may be one of several mechanisms driving the bathymetric faunal break observed in gobies and other reef fishes.

Glacioeustatic sea-level fluctuations are one possible mechanism that could initiate habitat transitions from shallow to deep reefs. After dramatic drops in sea levels during glacial cycles, subsequent rises in sea level would cause shallow-reef fauna to move with the changing seas, ultimately abandoning the areas occupied during low sea level stands in favour of warmer shallow water. Similarly, increases in sea surface temperature alone (in the absence of corresponding sea-level fluctuations) may cause species to initially move deeper in the water column to avoid thermal stress, and subsequently vacate deep-reef habitats when temperatures cool. In both scenarios, abandoned deep-reef habitats would then represent underutilized niches available to species that are able to stay deep and adapt to the new deep-reef conditions. Thus, under both scenarios we would expect to see shallow-to-deep habitat transitions immediately following periods where sea level fell dramatically and subsequently rose in a short time period, or where temperatures rose dramatically and subsequently dropped. When our estimates of habitat transition times are plotted against sea-level fluctuations and global temperatures (Fig. S1, Supporting information), our age estimates of deep-reef invasions overlap broadly with dramatic sea level drops at 30, 10 and 0–4 Ma, and also with abbreviated warming-to-cooling climate cycles at 26–28 and 13–18 Ma. However, the wide ranges in our estimates of transition times obscure any obvious causative relationship between deep-reef invasions, sea level and global climate.

In summary, the recent resurgence in the reconnaissance of Caribbean deep reefs, driven in large part by *DROP*, is further illuminating a true, deep-reef, Caribbean fish fauna and has enabled the discovery of numerous new species (Van Tassell *et al.* 2012; Baldwin & Robertson 2013, 2014, 2015; Baldwin & Johnson 2014; Tornabene *et al.* 2016). The deep-reef gobies investigated here are a major component of this deep-reef fish fauna, which collectively represent fishes that are ecologically divergent from their shallow-water counterparts. The deep-reef Caribbean goby fauna formed via several independent invasions from shallow reefs during the Miocene and Pliocene. While our estimates of the dates of each of these invasions are significantly different from each other, the habitat transitions can be broadly grouped into two main phases (Figs 3 and 4), each of which was followed by subsequent adaptive radiations on deep reefs. The initial phase occurred *c.* 10–30 Ma and involved independent, near-simultaneous shallow-to-deep transitions in the *Priolepis* lineage and the *Microgobius* group. Both of these lineages underwent subsequent diversifications as hovering, school-forming gobies on deep reefs. The second broad period of deep-reef radiation occurred within the last 10 Myr and included predominately demersal species that inhabit sand-rubble substrates—*C. curasub* and, possibly, the *Nes* clade, although the ancestors of the latter may instead have invaded during the first phase. It should be noted that while our taxon sampling of Caribbean gobies is robust (91% of Caribbean genera; 92% of Gobiosomatini genera; 90% of non-Gobiosomatini genera), our sampling across the entire Gobiidae is much more limited. A more comprehensive sampling across the gobiid phylogeny could affect the inferred phylogenetic position and divergence-time estimates of the deep-reef species that have Indo-Pacific relatives (i.e. the *Priolepis* lineage). In addition, continued reconnaissance of deep reefs both within the Caribbean and worldwide may reveal more undescribed mesophotic gobies, which could result in several phylogenetic patterns. New deep-reef species could potentially be (i) imbedded in our deep-reef clades, resulting in no change in our hypotheses of shallow-to-deep transitions; (ii) new deep-reef species could be resolved as a basal member of the deep-reef clades here, which could push estimates of the shallow-to-deep transitions towards the past; (iii) new deep-reef species could represent independent shallow-to-deep transitions beyond those identified in this study and could require additional hypotheses regarding the mechanisms driving habitat shifts. Given the well-known ability for gobies to rapidly exploit novel microhabitats and diversify via niche partitioning (e.g. Rüber *et al.* 2003; Yamada *et al.* 2009; Tornabene *et al.* 2013), it should come as no surprise that multiple independent lineages of gobies have successfully radiated into deep reefs throughout the last 30 Myr and contributed substantially to the formation of a true deep-reef fauna in the Caribbean. As exploration of the twilight zone continues, future studies will investigate whether other major deep-reef lineages display patterns of ecological and taxonomic diversification that mirror those in gobies, lending insights into the evolutionary origins of the deep-reef community as a whole.

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L.T., J.V.T., D.R.R. and C.C.B. designed the research and wrote the manuscript. L.T. collected molecular data and performed phylogenetic analyses. L.T., J.V.T., D.R.R. and C.B. collected depth and ecological data, which was analysed by L.T. and D.R.R. C.B., D.R.R. and J.V.T. collected specimens.

Data accessibility

1. Voucher specimens of deep-reef species are deposited at the Smithsonian Institution National Museum of Natural History.
2. Nucleotide alignment, BEAST2.xml file and file containing post-burn-in trees are deposited on Dryad. doi:10.5061/dryad.c3k7s
3. All novel DNA sequences generated from this study are deposited in GenBank.

Supporting information

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

Fig. S1 Age estimates of habitat transitions (see Figs 5 and 6) plotted alongside sea-level estimates (modified from Haq *et al*. 1987) and global temperatures (modified from Zachos *et al*. 2001).