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After continents divide: comparative phylogeography of reef fishes from the Red Sea and Indian Ocean

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ABSTRACT

Aim The Red Sea is a biodiversity hotspot characterized by a unique marine fauna and high endemism. This sea began forming *c.* 24 million years ago with the separation of the African and Arabian plates, and has been characterized by periods of desiccation, hypersalinity and intermittent connection to the Indian Ocean. We aim to evaluate the impact of these events on the genetic architecture of the Red Sea reef fish fauna.

Location Red Sea and Western Indian Ocean.

Methods We surveyed seven reef fish species from the Red Sea and adjacent Indian Ocean using mitochondrial DNA cytochrome *c* oxidase subunit I and cytochrome *b* sequences. To assess genetic variation and evolutionary connectivity within and between these regions, we estimated haplotype diversity (h) and nucleotide diversity (π), reconstructed phylogenetic relationships among haplotypes, and estimated gene flow and time of population separation using Bayesian coalescent-based methodology.

Results Our analyses revealed a range of scenarios from shallow population structure to diagnostic differences that indicate evolutionary partitions and possible cryptic species. Conventional molecular clocks and coalescence analyses indicated time-frames for divergence between these bodies of water ranging from 830,000 years to contemporary exchange or recent range expansion. Colonization routes were bidirectional, with some species moving from the Indian Ocean to the Red Sea compared with expansion out of the Red Sea for other species.

Main conclusions We conclude that: (1) at least some Red Sea reef fauna survived multiple salinity crises; (2) endemism is higher in the Red Sea than previously reported; and (3) the Red Sea is an evolutionary incubator, occasionally contributing species to the adjacent Indian Ocean. The latter two conclusions – elevated endemism and species export – indicate a need for enhanced conservation priorities for the Red Sea.

Keywords
Coalescent, cryptic speciation, dispersal, genealogical concordance, gene flow, mitochondrial DNA, vicariance.

INTRODUCTION

The Red Sea is a deep (maximum depth: 2920 m) and narrow (maximum width: 350 km) body of water extending 2270 km from 30° N in the Gulf of Suez to 13° N in the Gulf of Aden. Although the sea began forming *c.* 24 million years ago (Ma) (i.e. late-Oligocene period) by the separation of the African and Arabian plates, the ocean environment that supports coral reefs originated in the early Pleistocene (*c.* 5 Ma; Siddall *et al.*, 2003; Bosworth *et al.*, 2005). The Red Sea, which now experiences minimal freshwater inflow and high rates of evaporation, is characterized by

Journal of Biogeography

pronounced north-to-south gradients in salinity (42‰ to 37‰), sea surface temperature (winter, 20–28 °C; summer, 26–32 °C), and nutrient concentration (low to high) (Raitsos *et al.*, 2011; Ngugi *et al.*, 2012). Oceanographic current patterns and climate in the southern Red Sea (and Gulf of Aden) are heavily influenced by the northern Indian Ocean monsoon system (Smeed, 1997, 2004; Biton *et al.*, 2010), which reverses wind circulation patterns in the boreal summer (Southwest Monsoon, April to October) compared to those in the winter (Northeast Monsoon, December to March; see Fig. 1).

Despite its peripheral location relative to the Indo-Pacific, the Red Sea is characterized by high biodiversity, including *c.* 300 reef-building corals (mostly species of *Acropora* and *Porites*; Sheppard & Sheppard, 1991; Riegl & Velimirov, 1994) and 1078 fish species (Golani & Bogorodsky, 2010), which represent key resources for coastal communities. Species richness appears to be highest in the central Red Sea, with marked decreases in species abundance and changes in species composition away from this area (DeVantier *et al.*, 2000). The Red Sea also harbours one of the highest degrees of endemism for marine organisms, making up 14% of fishes (Randall, 1994), 33% of crustaceans, 15% of echinoderms, and up to 25% of corals (Cox & Moore, 2000). Endemism can be even higher for some taxa, reaching 50% in the butterflyfishes (Roberts *et al.*, 1992).

Although the evolutionary processes driving high rates of endemism are unclear, the narrow (18 km) and shallow (137 m) strait of the Bab al Mandab, the only connection with the Indian Ocean, is likely to have played a key role (Klausewitz, 1989). The Red Sea was repeatedly isolated during Pleistocene glacial cycles when the sea level lowered as much as 120 m; whether this was achieved through physical isolation or the restriction of oceanic flow associated with elevated salinity and temperature remains contentious (Siddall *et al.*, 2003; Bailey, 2009). Moreover, cold-water welling up off Somalia, which precludes reef formation on the north-east African and southern Arabian coasts, is likely to reinforce this isolation (Smeed, 1997; Kemp, 1998, 2000). Some authors believe that the Bab al Mandab no longer acts as a physical barrier to dispersal but that an ecological barrier lies within the Red Sea (Ormond & Edwards, 1987). Roberts *et al.* (1992) suggested that a turbid-water region south of 20° N in the Red Sea may limit larval dispersal, a

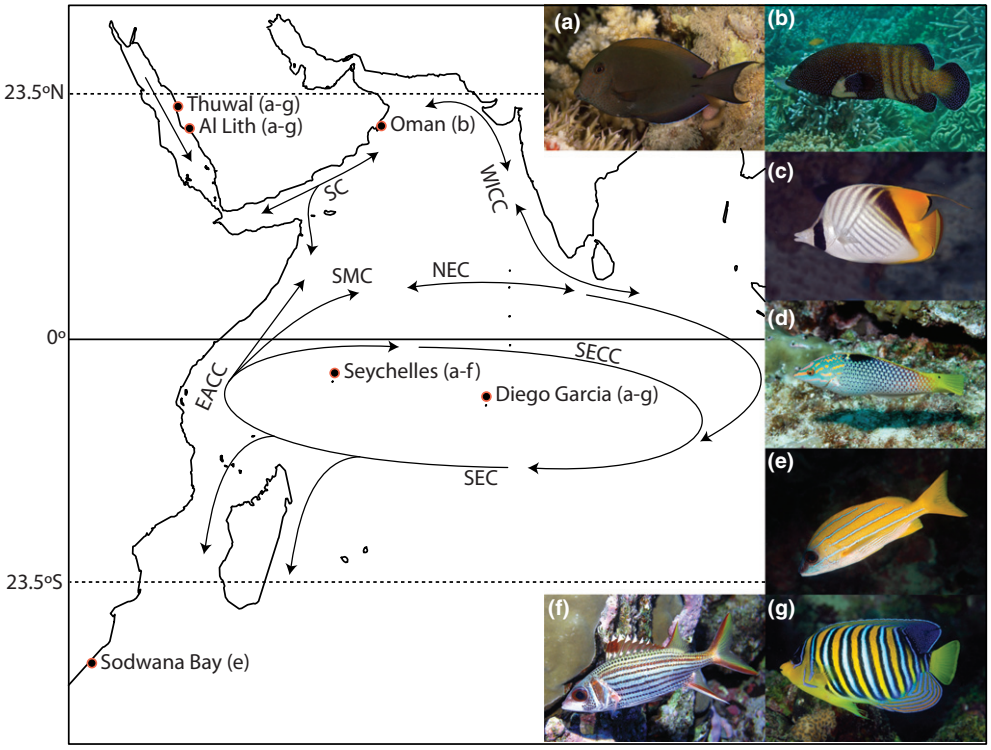

Figure 1 Scaled map indicating collection sites for all seven reef fish species (a, *Acanthurus nigrofuscus*; b, *Cephalopholis argus*; c, *Chaetodon auriga*; d, *Halichoeres hortulanus*; e, *Lutjanus kasmira*; f, *Neoniphon sammara*; g, *Pygoplites diacanthus*) sampled in the Red Sea and the Western Indian Ocean. Black lines with arrows show the major current systems flowing through each body of water (abbreviations: EACC, East African Countercurrent; NEC, Northwest Monsoon Current; SC, Somali Current; SEC, South Equatorial Current; SECC, South Equatorial Countercurrent; SMC, Southwest Monsoon Current; WICC, Western Indian Coastal Current). Note the reversing circulation of the SC (from northward to southward), the SMC (from westward to the eastward NEC), the WICC (from eastward to westward), and the current flowing into the Red Sea from the Gulf of Aden (compared with out of the Red Sea and into the Gulf of Aden) during the Northeast Monsoon season (December to March). Site-specific samples sizes are provided in Table 1. (Photo credits: M.L. Berumen, S. Moldzio and L.A. Rocha.)

hypothesis which is supported by the presence of a number of species in the northern/central Red Sea and the Gulf of Aden (just outside the Red Sea) that are absent from the southern Red Sea.

Despite being acknowledged as a biodiversity hotspot for coral reef fishes based on research dating back more than 200 years (e.g. Forsskål, 1775), little work has been conducted in the Red Sea using modern genetic techniques. Studies in this region tend to focus on the biogeography and community structure of the more iconic (and endemic) shore fish fauna (e.g. family Chaetodontidae; Righton *et al.*, 1996). The majority of genetic studies on reef fishes have been restricted to the Gulf of Aqaba and northern Red Sea (Hassan *et al.*, 2003; Kochzius & Blohm, 2005; but see Froukh & Kochzius, 2007), and few of these considered the connections between widespread taxa and other biogeographical provinces, particularly the Indian Ocean (Froukh & Kochzius, 2008).

Peripheral reef habitats such as the Red Sea, which forms the north-westernmost extension of the Indian Ocean, are typically considered to be biodiversity sinks that receive species from elsewhere but rarely export them (Briggs, 1999). The accepted paradigm is therefore that biogeographical sinks are 'evolutionary graveyards' that do not contribute to biodiversity at neighbouring sites. Recent research on reef fish and invertebrates, however, demonstrate that peripheral regions, such as the Hawaiian Archipelago and the Marquesas Islands, may act as both a sink and a source, contributing unique genetic lineages to other regions of the Indo-Pacific (Gaither *et al.*, 2010, 2011; DiBattista *et al.*, 2011; Eble *et al.*, 2011; Skillings *et al.*, 2011).

Our first aim is to assess connections between fauna in the Red Sea and the adjacent Western Indian Ocean (WIO) using a molecular genetic approach. The WIO forms a biogeographical subdivision of the tropical Indo-Pacific stretching from East Africa to the Chagos Ridge in the centre of the Indian Ocean (Sheppard, 2000; Briggs & Bowen, 2012). Phylogeographical inferences are strengthened by congruence among multiple species and genes, and so our study considers seven species of reef fish with widespread distributions, using two mitochondrial DNA (mtDNA) markers.

Our second aim is to assess whether sea level changes have influenced extant biodiversity by estimating migration rates and divergence times of reef fishes in the Red Sea and WIO. Such analyses will allow us to discriminate between the following scenarios: (1) Red Sea populations represent long-isolated relicts derived from the WIO, which implies gene flow was absent over the last 5 Myr; (2) Red Sea populations have been isolated from the WIO over evolutionary intervals but with recurrent gene flow; or (3) Red Sea populations are the result of recent colonization from the WIO, since the Last Glacial Maximum *c.* 20,000 years ago (Siddall *et al.*, 2003; Bailey, 2009). This dataset provides an unprecedented opportunity to assess the relationships between two Indian Ocean biogeographical provinces, and thereby illuminate evolutionary processes that are the wellspring of Red Sea biodiversity.

MATERIALS AND METHODS

Sample collection

Reef fish were collected while SCUBA diving or snorkelling at depths of 1–40 m between 2002 and 2011 (Fig. 1, Table 1). Seven reef fish species were targeted: the brown surgeonfish, *Acanthurus nigrofuscus* (Forsskål, 1775); the peacock hind, *Cephalopholis argus* Schneider, 1801; the threadfin butterflyfish, *Chaetodon auriga* Forsskål, 1775; the checkerboard wrasse, *Halichoeres hortulanus* (Lacepède, 1801); the bluestripe snapper, *Lutjanus kasmira* (Forsskål, 1775); the Sammara squirrelfish, *Neoniphon sammara* (Forsskål, 1775); and the regal angelfish, *Pygoplites diacanthus* (Boddaert, 1772). These species were chosen because they have wide Indo-Pacific distributions, are abundant, represent a diversity of taxonomic families, and can be unequivocally identified in the field. Each species was sampled at two locations (Thuwal and Al Lith) off the coast of the Kingdom of Saudi Arabia (KSA) in the central Red Sea, and at one to three sites in the WIO (oceanic sites: Diego Garcia in the Chagos Archipelago and the Republic of Seychelles; coastal sites: Sodwana Bay, South Africa and Al Hallaniyat, Sultanate of Oman). Because some of the collections were opportunistic, not every species could be sampled at every location (Fig. 1, Table 1).

Mitochondrial DNA sequencing

Tissue samples were preserved in salt-saturated DMSO (Seutin *et al.*, 1991). Total genomic DNA was extracted using the 'HotSHOT' protocol (Meeker *et al.*, 2007) and subsequently stored at −20 °C. Fragments of mtDNA from the cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) genes were amplified using either previously published primers or modified primers designed for individual species (Table 1). These two markers were chosen because they: (1) are easy to amplify in most fish; (2) are generally variable at the population level; (3) facilitate comparisons with published sequences; and (4) have had molecular clock rates estimated based on reef fishes (Bowen *et al.*, 2001; Lessios, 2008; Reece *et al.*, 2010). Polymerase chain reaction (PCR) was carried out for all species as described in DiBattista *et al.* (2012a), with optimal annealing temperatures listed in Table 1. All samples were sequenced in the forward direction (and reverse direction for unique or questionable haplotypes) with fluorescently labelled dye terminators (BigDye version 3.1, Applied Biosystems, Foster City, CA, USA) and analysed using an ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequences were aligned, edited and trimmed to a uniform length using Geneious Pro 4.8.4 (Drummond *et al.*, 2009); unique mtDNA haplotypes were deposited in GenBank (accession numbers: KC187734–KC188056).

Table 1 Study species, number of specimens, fragment length, primers used, and annealing temperatures for mitochondrial DNA cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) genes. DNA sequences from each primary collection location (Al Lith, Thuwal, Diego Garcia, and the Republic of Seychelles; see text) are described, along with collections made opportunistically at additional locations in the Western Indian Ocean (WIO). All haplotypes are available online in GenBank (accession numbers: KC187734–KC188056).

	Molecular sequence data								
			Red Sea		WIO				
Species	DNA fragment	Fragment length (bp)	Al Lith	Thuwal	Diego Garcia	Seychelles	Other sites (*n*)	Primer set	Annealing temp. (°C)
Acanthurus nigrofuscus (brown surgeonfish)	COI	634	22	27	31	31	–	Fish F2–Fish R2 (1)	50
	cyt *b*	683	22	28	31	30	–	Cyb9–Cyb7 (2,3)	58
Cephalopholis argus (peacock hind)	COI	537	26	19	24	10	Oman (8)	Fish F2–Fish R2 (1)	56
	cyt *b*	618	27	22	32	13	Oman (9)	CB6F–CB6R (4)	54
Chaetodon auriga (threadfin butterflyfish)	COI	625	27	20	33	30	–	Fish F2–Fish R2 (1)	52
	cyt *b*	670	27	20	33	30	–	Cyb9–Cyb7 (2,3)	56
Halichoeres hortulanus (checkerboard wrasse)	COI	589	25	27	20	28	–	Fish F2–Fish R2 (1)	50
	cyt *b*	692	25	27	27	22	–	Cyb9–Cyb7 (2,3)	50
Lutjanus kasmira (bluestripe snapper)	COI	606	21	22	33	20	Sodwana Bay (34)	Fish F2–Fish R2 (1)	56
	cyt *b*	475	23	23	34	19	Sodwana Bay (34)	H15020–Cyb5 (5,3)	48
Neoniphon sammara (Sammara squirrelfish)	COI	611	20	31	30	28	–	Fish F2–Fish R2 (1)	50
	cyt *b*	508	20	31	29	38	–	NSAFOR4–NSAREV4*	60
Pygoplites diacanthus (regal angelfish)	COI	634	24	23	33	–	–	Fish F2–Fish R2 (1)	50
	cyt *b*	640	24	23	32	–	–	PydCytbF3–PydCytbR4*	50

*We designed two novel primer sets to amplify and sequence cyt *b* for *Neoniphon sammara* and *Pygoplites diacanthus*. Their sequences were as follows:
NSAFOR4: 5′-TGC CGT GAC GTA AAC TAT GG-3′; NSAREV4: 5′-TGA AGT TGT CGG GAT CTC CT-3′; PydCytbF3: 5′-ATG GCA AAC TTA CGC AAA ACC-3′; PydCytbR4: 5′-GGC TGG TGT GAA GTT GTC-3′.
References: (1) Ward *et al.*, 2005; (2) Song *et al.*, 1998; (3) Taberlet *et al.*, 1992; (4) Gaither *et al.*, 2010; (5) Meyer, 1994.

Genetic diversity and population structure

Arlequin 3.5 (Excoffier *et al.*, 2005) was used to calculate haplotype and nucleotide diversity (h and π, respectively), and to test for population structure among sampling sites for each species and molecular marker (i.e. 14 total datasets). These analyses were repeated with all Red Sea or WIO samples pooled into two separate regions. Despite the difference in the geographical scale of sampling (Red Sea sites, *c.* 300 km; WIO sites, *c.* 1000s of km), preliminary work suggests that the Red Sea haplotypes at Thuwal and Al Lith are consistent with those sampled up to 1200 km north (J.D.D., unpublished data), indicating unbiased estimates of genetic diversity within our study range. Because jModelTest 0.1.1 (Posada, 2008) converged on different models of nucleotide sequence evolution among datasets, we calculated global and pairwise Φ_{ST} values based on a HKY model of mutation (Hasegawa *et al.*, 1985). We also ran conventional frequency-based F_{ST}, but the absolute values changed little and relative values did not change at all; we have therefore elected to report pairwise Φ_{ST}. Global Φ_{ST} was estimated using analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992); deviations from null distributions were tested with nonparametric permutation procedures (n = 99,999). We controlled for false discovery rate with the method of Narum (2006), and negative pairwise Φ_{ST} values were converted to zeros. To facilitate comparisons among species, an additional diversity measure – Jost's D (Jost, 2008) – was estimated using spade 1.0 (Chao *et al.*, 2008).

The evolutionary relationship among COI or cyt *b* haplotypes was resolved for each species with unrooted networks constructed with the program network 4.5.1.0 (http://www.fluxus-engineering.com/network_terms.htm) using a median-joining algorithm and default settings (as per Bandelt *et al.*, 1999).

IMa2 analysis

We calculated the effects of time and gene flow on genetic divergence between populations using Bayesian coalescent-based estimation with IMa2 8.26.11 (Hey & Nielsen, 2007; Hey, 2010). Using *F*-statistics we determined that samples within regions were not significantly different for all seven species after correction for multiple comparisons. We therefore pooled the Red Sea sites together and the WIO sites together, for comparisons between regions for each species and molecular marker.

The isolation-with-migration model implemented in IMa2 assumes that two populations of effective size N_1 and N_2 diverged from an ancestral population (of effective size N_a)

at time t, and then exchanged migrants at rates m_1 and m_2. We therefore estimated the time since initial separation or last major colonization event (t), effective population size (N_e), and the proportion of migrants arriving into a population per generation (m); all demographic parameters were scaled by mutation rate.

Mutation rates calibrated in other reef fish based on the closure of the Isthmus of Panama range from 1% to 2% per million years for COI and cyt *b* (Bowen *et al.*, 2001; Lessios, 2008; Reece *et al.*, 2010). We used a conservative estimate of 1.3×10^{-8} mutations per site per year for both markers (see Lessios, 2008) under a HKY model and a 0.25 inheritance scalar appropriate for mtDNA. An MCMC chain with a length of 1,000,000 sampled every 100 generations with 10% burn-in was used to estimate parameters for each species–gene combination. Five independent runs were computed to ensure convergence. The independent runs were subsampled and combined using the 'L' mode of IMa2, and the median values of the parameter distributions for the combined runs are presented here. For *N. sammara* (COI) and *P. diacanthus* (COI and cyt *b*), which shared almost no haplotypes between regions, prior values of m in both directions were set to zero. Although we regard all absolute parameter estimates with caution given that our data consist of two linked loci, and we apply mutation rates calibrated in other reef fishes, relative comparisons among species are likely to be robust to such approximations (Karl *et al.*, 2012).

RESULTS

Genetic diversity and population structure

COI and cyt *b* sequence data revealed divergent patterns of genetic diversity and population structure among the seven sampled reef fish species. Haplotype (h) and nucleotide (π) diversity was higher in four out of the seven species in the WIO than in the Red Sea for COI, and in five out of the seven species for cyt *b* when populations within each region were pooled (Fig. 2, and see Appendix S1 in Supporting Information). This trend cannot be explained by a greater sampling effort in the WIO, given that species with comparable sample sizes for each region, such as *Cephalopholis argus* and *P. diacanthus*, still had lower genetic diversity in the Red Sea.

AMOVA supported the geographical grouping of sites into Red Sea and WIO regions (Table 2). Although there was some variability in genetic differentiation among sampling sites between regions (Appendix S2), six out of the seven species showed significant genetic structure for at least one of the molecular markers (Table 2).

Population pairwise tests were significantly different in 22 (for COI) or 16 (for cyt *b*) out of 47 comparisons (all $P < 0.01$); all significant comparisons were between regions rather than between sites within regions, and ranged from 0.07 to 0.67 for Φ_{ST} and 0.05 to 1.00 for Jost's D (Fig. 3, Appendix S2). Estimates of genetic differentiation across all species were correlated between molecular markers (nonparametric Spearman's rank correlation: Φ_{ST}, $r = 0.77$, $P < 0.001$, $n = 47$; Jost's D, $r = 0.57$, $P < 0.001$, $n = 47$), although the larger spread of Jost's D values than Φ_{ST} values is probably related to the former not being constrained by within-site heterozygosity. Pairwise genetic differentiation-based Φ_{ST} and Jost's D were also significantly correlated across all datasets (nonparametric Spearman's rank correlation coefficient: COI, $r = 0.67$, $P < 0.001$; cyt *b*, $r = 0.83$, $P < 0.001$).

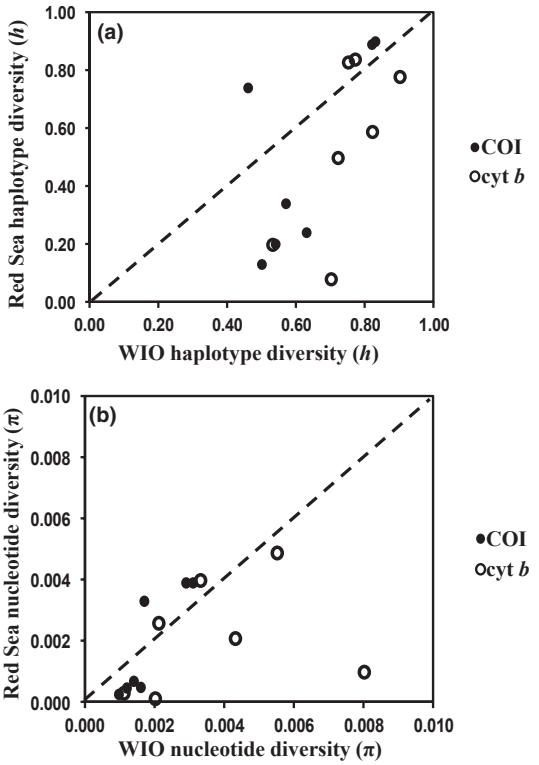

Figure 2 The relationship between (a) haplotype diversity (h) or (b) nucleotide diversity (π) estimated for mitochondrial DNA cytochrome *c* oxidase subunit I (COI; black filled circles) and cytochrome *b* (cyt *b*; open circles) genes in the Red Sea versus Western Indian Ocean (WIO) populations of species considered in this study. The black dashed line represents a line of unity, which is the point at which genetic diversity estimates in the Red Sea and WIO are equal within a species. Data points above the line of unity indicate greater genetic diversity in the Red Sea, whereas points falling below the line indicate greater genetic diversity in the WIO.

As expected from the Φ_{ST} values, the median-joining networks show more shared COI or cyt *b* haplotypes between collection sites within the Red Sea and WIO than between these regions (Fig. 4). The only exception to this pattern was the high proportion of haplotypes shared between Al Lith or Thuwal (Red Sea) and Oman (WIO) for *Cephalopholis argus*. Even though our comparisons between Oman and other sites should be viewed with caution, given that these are based on data from only a single species (*C. argus*) with a low sample size ($n = 8$ or 9),

Table 2 Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) comparing variation between the Red Sea and Western Indian Ocean populations of reef fish based on mitochondrial DNA cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) genes. Site-specific samples sizes are shown in Table 1.

		Percentage variation					
Species	DNA fragment	Within populations	Between populations	Between regions	Overall Φ_{ST}	*P*	Jost's *D* (SE)
Acanthurus nigrofuscus	COI	76.58	0.25	23.18	0.23	< 0.001	0.59 (0.051)
(brown surgeonfish)	cyt *b*	87.96	−0.64	12.68	0.12	< 0.001	0.21 (0.087)
Cephalopholis argus	COI	72.45	−0.31	27.86	0.28	< 0.001	0.13 (0.088)
(peacock hind)	cyt *b*	77.59	1.02	21.39	0.22	< 0.001	0.13 (0.060)
Chaetodon auriga	COI	80.35	−1.80	21.45	0.20	< 0.001	0.087 (0.041)
(threadfin butterflyfish)	cyt *b*	98.48	−0.44	1.96	0.015	0.12	0.021 (0.022)
Halichoeres hortulanus	COI	97.30	2.60	0.10	0.027	0.071	0.041 (0.019)
(checkerboard wrasse)	cyt *b*	95.44	1.08	3.48	0.046	0.045	0.078 (0.088)
Lutjanus kasmira	COI	100.11	−1.30	1.20	0	0.83	0.006 (0.023)
(bluestripe snapper)	cyt *b*	98.88	−0.46	1.58	0.011	0.23	0.090 (0.059)
Neoniphon sammara	COI	66.03	−0.33	34.30	0.34	< 0.001	0.68 (0.038)
(Sammara squirrelfish)	cyt *b*	70.28	−0.69	30.41	0.30	< 0.001	0.59 (0.060)
Pygoplites diacanthus	COI	30.61	−1.17	70.57	0.69	< 0.001	0.61 (0.015)
(regal angelfish)	cyt *b*	65.00	−2.30	37.30	0.35	< 0.001	0.60 (0.027)

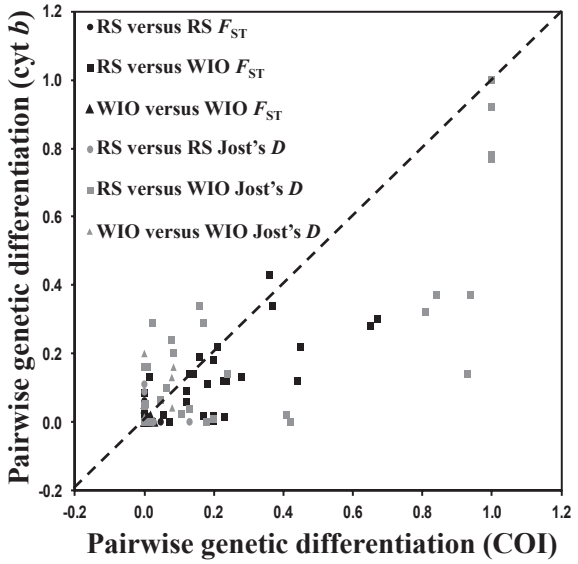

Figure 3 The relationship between mitochondrial DNA cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) estimates of pairwise genetic differentiation for the seven species of reef fish, based on comparisons among Red Sea (RS, represented by circles), Western Indian Ocean (WIO; represented by triangles), and Red Sea versus WIO sites (represented by squares). Estimates of both Φ_{ST} (black symbols) and Jost's *D* (grey symbols) are presented here. The black dashed line represents a line of unity, which is the point at which pairwise genetic differentiation estimates between two study sites are equal for each molecular marker. Data points below the line of unity indicate greater genetic differentiation based on COI, whereas points falling above the line indicate greater genetic differentiation based on cyt *b*.

some endemic Red Sea fauna do extend to the Omani coast (e.g. *Cirrhitus spilotoceps*, M.R.G. & J.E. Randall, Bishop Museum, unpublished data).

IMa2 analysis

The estimated times since initial separation between the Red Sea and WIO populations for the seven reef fish species ranged from *c.* 21,000 to 830,000 years (Table 3). Recent separations of < 100,000 years were apparent for *Chaetodon auriga* and *H. hortulanus*, older separations of 100,000–300,000 years were apparent for *A. nigrofuscus*, *Cephalopholis argus*, *L. kasmira* and *N. sammara*, and finally *P. diacanthus* populations have been isolated for 660,000–830,000 years. Of the older separations, *L. kasmira* was characterized by high subsequent gene flow, whereas gene flow was restricted for *N. sammara* (and *P. diacanthus*); these two species also have the highest level of divergence between the Red Sea and WIO based on Φ_{ST} (Table 2). Differences among species in both the timing of initial divergence and subsequent migration rates reveal considerable variation in the link between Red Sea and WIO populations.

The direction of migration varied among species. For example, a higher proportion of migrants moved from the WIO into the Red Sea for *H. hortulanus*, whereas *L. kasmira* moved predominantly out of the Red Sea (Table 3). For the remaining species, gene flow was low in both directions, or driven by differences in effective population size, indicating no bias in effective migration between regions.

DISCUSSION

This study demonstrates barriers to gene flow between the Red Sea and WIO for some reef fish species, but an apparent lack of phylogeographical breaks for others, which may reflect the volatile geological history of the Red Sea region.

The Red Sea is a marginal water mass whose movement in the upper layers is driven by the summer and winter

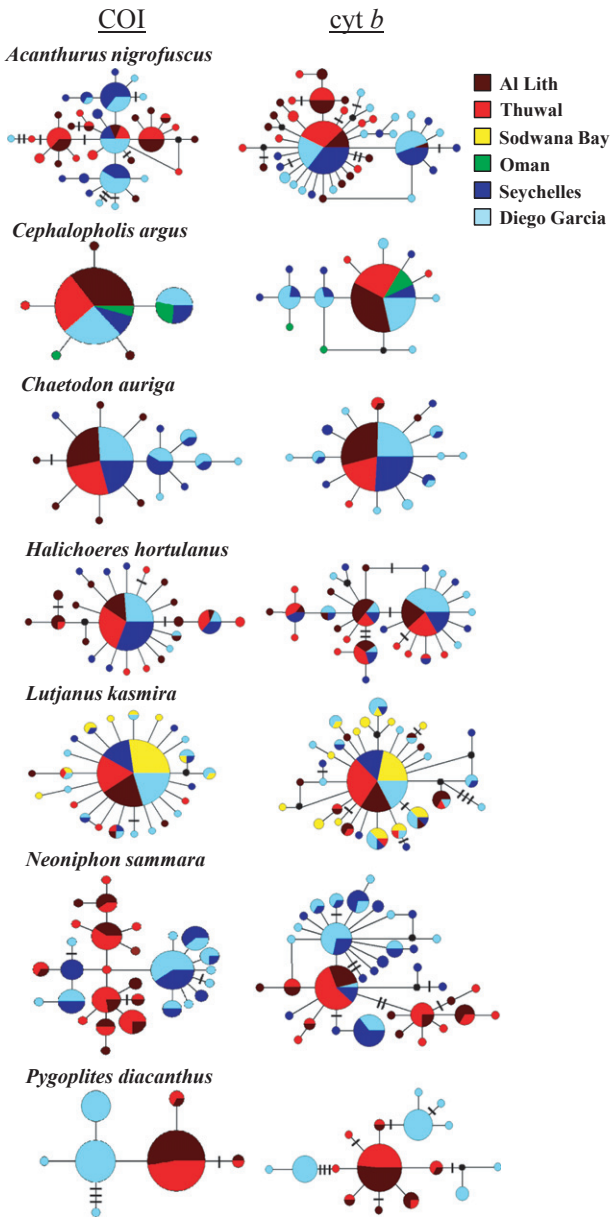

Figure 4 Median-joining networks showing relationships among mitochondrial DNA cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) haplotypes for each study species (*Acanthurus nigrofuscus*; *Cephalopholis argus*; *Chaetodon auriga*; *Halichoeres hortulanus*; *Lutjanus kasmira*; *Neoniphon sammara*; *Pygoplites diacanthus*) collected in the Red Sea (Al Lith and Thuwal) and the Western Indian Ocean (Diego Garcia, Oman, Seychelles and Sodwana Bay). Each circle represents a unique haplotype and its size is proportional to its total frequency. Branches or black cross-bars represent a single nucleotide change, small black circles represent missing haplotypes, and colours denote collection location as indicated by the embedded key.

monsoons acting through a restricted connection with the adjacent Gulf of Aden (Siddall *et al.*, 2004; Biton *et al.*, 2008). During each glacial maximum of the Pleistocene, the last characterized by a 120-m drop in sea level only 20,000 years ago, the inflow pattern and exchange of surface water was limited, owing to the shallow sill at the Bab al Mandab – the only natural gateway into the Red Sea (Siddall *et al.*, 2003). As a result, throughout these periods of isolation, increased evaporation may have raised temperature and salinity levels higher than most reef fishes can tolerate (e.g. > 50‰; Biton *et al.*, 2008), resulting in periods of reduced planktonic (i.e. larval) development (Hemleben *et al.*, 1996), and causing mass extirpation within the Red Sea (Sheppard *et al.*, 1992; but see Klausewitz, 1989).

In addition to intermittent historical barriers created by Pleistocene glacial cycles, contemporary barriers exist. The lack of coral habitat along the 2200-km coastline from Djibouti to southern Somalia may inhibit gene flow between the Red Sea and WIO by limiting opportunities for stepping-stone dispersal (Kemp, 1998). Within the Red Sea, the extensive turbid-water region south of 20° N may also inhibit larval dispersal or settlement (Ormond & Edwards, 1987; Roberts *et al.*, 1992). The long-term persistence and age of these contemporary barriers, however, is uncertain.

Most genetic work on reef fishes within the Red Sea has focused on the differentiation of fauna between the Gulf of Aqaba and northern Red Sea, with some notable exceptions. Froukh & Kochzius (2008) identified a damselfish in the Red Sea (*Chromis viridis*) as being distinct from conspecifics in Indonesia and the Philippines based on mtDNA sequences. Similar research on marine invertebrates (*Acanthaster planci*: Benzie, 1999; *Scylla serrata*: Fratini & Vannini, 2002) support a genetic distinction of the Red Sea populations. In contrast, Kochzius & Blohm (2005) found no mtDNA differentiation between lionfish (*Pterois miles*) populations in the Red Sea and Indian Ocean.

Five of the seven species we examined were genetically differentiated between the Red Sea and WIO based on AMOVA and median-joining networks. *Halichoeres hortulanus* and *L. kasmira* had minimal or inconsistent genetic differentiation, as well as extensive mixing of haplotypes within and between regions. *Acanthurus nigrofuscus*, *Cephalopholis argus* and *Chaetodon auriga* had modest differentiation between regions with pronounced separation of peripheral haplotypes, but shared a common haplotype among all sampling sites. *Neoniphon sammara* and *Pygoplites diacanthus* had fixed differences between regions.

Variability in genetic signatures can occur even among closely related species (Rocha *et al.*, 2002; Gaither *et al.*, 2010; DiBattista *et al.*, 2012b) and may be related to innate differences in life history or ecological preferences, although these widely distributed species are all presumably capable of long-distance dispersal (e.g. Eble *et al.*, 2009, 2011; Gaither *et al.*, 2010, 2011) based on available estimates of pelagic larval duration (range: 24–48 days; Thresher & Brothers, 1985; Victor, 1986; Wilson & McCormick, 1999) and longitudinal range size (range: 20,063–21,689 km; Randall, 1999, 2005). Indeed, our study species cover a wide spectrum of dietary modes ranging from herbivory (*A. nigrofuscus*) to specialist feeding on sessile or mobile invertebrates (*Chaetodon auriga*,

Table 3 Estimates of time in years (t) since initial separation, effective migration rate ($2N_em$), effective population sizes (N_e), and mutation-scaled migrations rates (m) between Red Sea (RS) and Western Indian Ocean (WIO) populations of seven reef fish species based on mitochondrial DNA cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) runs in IMa2 (Hey & Nielsen, 2007). Abbreviations: NC, no convergence. Inequalities: posterior probability densities rise to a plateau, so that all estimates larger than the shown value have the same approximate posterior probability.

Species	DNA fragment	Initial separation in years (t)	Effective migration rate ($2N_em$)		Effective population size (N_e)		Migration (m)	
			WIO to RS	RS to WIO	WIO	RS	WIO to RS	RS to WIO
Acanthurus nigrofuscus (brown surgeonfish)	COI	105,000	1.21	0.09	25.10	50.70	0.03	0.01
	cyt *b*	79,000	3.92	0.11	52.75	85.25	0.02	0.001
Cephalopholis argus (peacock bind)	COI	> 121,000	NC	0.73	0.25	NC	21.05	1.45
	cyt *b*	> 212,000	0.01	NC	NC	0.60	0.01	6.27
Chaetodon auriga (threadfin butterflyfish)	COI	26,800	0.08	0.13	6.60	3.80	0.01	0.01
	cyt *b*	30,700	0.03	4.85	34.65	1.65	0.01	0.07
Halichoeres hortulanus (checkerboard wrasse)	COI	26,500	1491.11	0.08	7.50	115.50	6.46	0.01
	cyt *b*	21,600	> 199.50	> 163.50	> 272.50	> 262.50	1.22	0.02
Lutjanus kasmira (bluestripe snapper)	COI	41,000	0.23	1282.22	104.50	7.50	0.02	6.14
	cyt *b*	155,000	15.01	104.61	88.10	3.10	2.82	0.50
Neoniphon sammara (Sammara squirrelfish)	COI	169,000	0.79	0.03	14.85	20.75	0.02	0.001
	cyt *b*	190,000	0.09	0.24	47.48	17.77	0.0025	0.0025
Pygoplites diacanthus (regal angelfish)	COI	831,000	0.01	0.04	4.20	1.40	0.01	0.01
	cyt *b*	> 662,000	0.06	0.04	8.88	11.63	0.0025	0.0025

H. hortulanus and *P. diacanthus*) to piscivory (*Cephalopholis argus*, *L. kasmira* and *N. sammara*). These species also display a variety of reproductive behaviours, ranging from dioecism (*L. kasmira*, *N. sammara*) with mate-pairing (*Chaetodon auriga*) or spawning aggregations (*A. nigrofuscus*) to protogyny (*Cephalopholis argus* and *H. hortulanus*). Given that there are no real unifying life-history features for this diverse group, we suspect that differences in ecological resilience to geological disturbance may have contributed to the range of colonization histories, although this will require further testing.

Considering the prevailing currents in the Indian Ocean, it is not surprising that sampling sites in the WIO were genetically similar to each other. The Chagos and Seychelles archipelagos are located in the South Equatorial Current, which flows from east to west. Both archipelagos are also heavily influenced by seasonal or permanent countercurrents (South Equatorial Countercurrent and East African Countercurrent, respectively; Fig. 1). The strong and variable water movement of the region has resulted in Diego Garcia, which is located at the southern end of the Chagos Archipelago, having faunal affinities with both the Indo-Polynesian and WIO provinces (Winterbottom & Anderson, 1997; Craig, 2008; Gaither *et al.*, 2011; Briggs & Bowen, 2012). The South African coastline is similarly well connected to the central Indian Ocean, being influenced by the warm Mozambique/Agulhas current (Lutjeharms, 2006), which facilitates unidirectional (north to south) transport of tropical fauna from other sites in the WIO.

There are several records of long-distance dispersal of tropical reef fish (e.g. *Chaetodon zanzibarensis* and *Ecsenius lineatus*) to the Arabian coastline during periods of upwelling, which indicate that larval transport from the WIO to this region and subsequent settlement are not precluded (Kemp, 2000). Although we only sampled a few specimens ($n = 9$) of a single species off the coast of Oman (*Cephalopholis argus*), these fish were not genetically distinct from conspecifics sampled at Diego Garcia (COI: $\Phi_{ST} = 0.029$, $P = 0.25$; cyt *b*: $\Phi_{ST} < 0.001$, $P = 0.50$) or the Seychelles (COI: $\Phi_{ST} < 0.001$, $P = 0.80$; cyt *b*: $\Phi_{ST} = 0.039$, $P = 0.20$).

Vicariance events and colonization history

Our mtDNA data provide evidence for three separate periods of colonization or export of propagules between the Red Sea and WIO (Table 3). First, Red Sea populations of *Chaetodon auriga* and *H. hortulanus* appear to derive from the WIO during or soon after the most recent glacial maximum (*c.* 21,000–31,000 years ago; but see Karl *et al.*, 2012). Second, population separations in *A. nigrofuscus*, *Cephalopholis argus* and *L. kasmira* pre-date the Last Glacial Maximum but include recurrent gene flow in most cases. Third, *N. sammara* and *P. diacanthus* represent long-isolated evolutionary lineages in the Red Sea. These final cases in particular indicate that some Red Sea residents survived the major temperature and salinity crises recorded 19,000, 30,000 and 450,000 years ago (Siddall *et al.*, 2003).

IMa2 analyses indicate bidirectional gene flow between the Red Sea and WIO, which is also apparent in the older history inscribed in haplotype networks (Fig. 4). Three cases provide especially strong inference: (1) in the COI and cyt *b* network for *A. nigrofuscus*, the central (ancestral) haplotype is observed primarily in the Indian Ocean, whereas the Red Sea haplotypes are peripheral; (2) in the COI network for *N. sammara*, the central haplotype is detected only in the Red Sea, with the Indian Ocean haplotypes peripheral; and (3) in

the cyt *b* network for *P. diacanthus*, the central haplotype is detected only in the Red Sea. Hence the networks for these three species indicate colonization into and out of the Red Sea, which supports the hypothesis that peripheral habitats can export biodiversity to the central Indo-Pacific.

Taxonomic considerations

Our genetic study highlights three interesting cases where the current classification of existing species may not reflect their evolutionary history. *Chaetodon auriga* is one of the most widespread butterflyfishes on the planet, with a distribution of *c.* 82.2 million km^2 across the tropical Indo-Pacific (Allen *et al.*, 1998). The original species description is from Red Sea specimens, which lack a dark spot on the margin of the soft dorsal fin, such that conspecifics outside the Red Sea were regarded as the subspecies *C. auriga setifer* (Bloch, 1795).

Although we did detect differences in mtDNA sequences between the Red Sea and WIO, these were only marginally significant. In addition, the most common haplotype was shared between the Red Sea and WIO at both COI and cyt *b*. Even though colour morphs do correspond to genetic partitions in some species (e.g. Craig & Randall, 2008; Drew *et al.*, 2008; Randall & Rocha, 2009), discordance between genetic divergence and coloration is well documented in reef fishes (Ramon *et al.*, 2003; Rocha, 2004; Messmer *et al.*, 2005), including butterflyfishes (family Chaetodontidae: McMillan & Palumbi, 1995) and their sister group, the angelfishes (family Pomacanthidae: Bowen *et al.*, 2006; DiBattista *et al.*, 2012a). For these reasons, we regard the Red Sea population as conspecific with all other populations of *Chaetodon auriga*, although the shallow but significant population genetic differentiation supports the subspecific status.

Two species in our study reveal a strikingly different pattern: *N. sammara* and *P. diacanthus* were characterized by high Φ_{ST} (or Jost's *D*) values relative to all other species and strong mtDNA differences between regions. For *P. diacanthus* in particular, Red Sea and WIO haplotypes are separated by at least three mutations at cyt *b* and one fixed mutation at COI, indicating isolation for several hundred thousand years. This genetic separation is matched by coloration differences between Red Sea and WIO populations (Allen *et al.*, 1998), indicating long-isolated populations that, unlike other examined species, failed to reconnect during interglacial periods. Notably, the species is absent from sites in the Arabian Sea, indicating geographical isolation (Kemp, 1998). While we know of no coloration or morphological differences in *N. sammara* that may indicate cryptic lineages, this possibility merits further investigation.

CONCLUSIONS

Comparative phylogeographical studies have done much to illuminate the evolutionary history of regional marine faunas (Avise, 1992; Lessios & Robertson, 2006; Kelly & Palumbi, 2010; Carpenter *et al.*, 2011; Toonen *et al.*, 2011; Ludt *et al.*, 2012). Here we provide the first multispecies comparison between Red Sea and Indian Ocean reef fishes, and find a spectrum of outcomes from recent gene flow to ancient evolutionary separations. Three broad conclusions are apparent. First, endemism and biodiversity are higher among Red Sea reef fishes than previously suspected (i.e. *N. sammara* and *P. diacanthus*), and ongoing studies will be likely to elevate these estimates. Second, some elements of the Red Sea fauna survived the salinity crises caused by late Pleistocene glaciations. This does not require continuous residence in the Red Sea, as persistence in the Gulf of Aden just outside the Red Sea remains a possibility. It seems unlikely, however, that a genetically distinct and cohesive fauna could survive in the Gulf without extensive admixture with other Indian Ocean populations. We therefore favour the explanation that Red Sea refugia existed during low sea level stands associated with glaciations. Third, peripheral habitats such as marginal seas and isolated archipelagos are not necessarily 'evolutionary graveyards'. Rather, our data indicate that the Red Sea is capable of exporting biodiversity to the broader Indo-Pacific, thus operating as a potential engine of evolutionary diversity in our oceans.

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SUPPORTING INFORMATION

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

Appendix S1 Mitochondrial DNA haplotype (*h*) and nucleotide diversity (π) for each species from each collection location.

Appendix S2 Population pairwise Φ_{ST} and Jost's *D* values for each species based on mitochondrial DNA sequences.

BIOSKETCH

The authors' interests are focused on illuminating the evolutionary processes that generate marine biodiversity. They have carried out phylogeographical surveys of over 20 reef fish species in the greater Indo-Pacific to test existing evolutionary models, resolve the life-history traits that influence dispersal and population separations in reef organisms, and inform marine conservation (e.g. defining the boundaries of marine protected areas).

Author contributions: J.D.D. conceived the ideas for this study, collected tissue samples and produced DNA sequences, analysed the data, and led the writing. In addition to contributing to writing, M.L.B., B.W.B., J.H.C. and M.T.C. collected tissue samples, M.R.G. and L.A.R. collected tissue samples and produced DNA sequences, J.A.E. produced DNA sequences, and D.J.S. implemented and interpreted coalescent analyses.

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