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Basin isolation and oceanographic features influencing lineage divergence in the humbug damselfish (*Dascyllus aruanus*) in the Coral Triangle

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ABSTRACT.—The Coral Triangle is a hotspot for marine species diversity as well as for intraspecific genetic diversity. Here, we used nuclear RAG2 and mitochondrial D-Loop genes to identify deep genetic divergence among *Dascyllus aruanus* (Linnaeus, 1758) populations across relatively short scales within the Coral Triangle. Mitochondrial clades different by >20 mutational steps were completely isolated from one another across the distance between Java and the Lesser Sunda Islands, and also showed frequency differences in the eastern and western Philippines. Evidence for population structure in the Sulu Sea and at the Lesser Sunda Islands was also identified. Our results suggest that the Sulu Sea Throughflow, Bohol Sea Throughflow, Indonesian Throughflow, signatures of extinction events from Pleistocene land barriers, and other past and present forces may be potential factors leading to lineage divergence of *D. aruanus*, and that these hypotheses should be tested in further studies.

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The Coral Triangle encompasses the greatest concentration of marine biodiversity on Earth (Rosen 1971, Stehli and Wells 1971, Sanciangco et al. 2013). This biogeographic pattern is typically hypothesized as an area of species overlap, refuge, origin, or accumulation (Palumbi 1996, Barber et al. 2011, Carpenter et al. 2011), although multiple processes likely contribute to elevated biodiversity in this region (Barber 2009, Bowen et al. 2013). Isolation of populations and subsequent speciation within the Coral Triangle are integral to some of these hypotheses (Palumbi 1994), and there are numerous examples of allopatric lineage diversification among marine populations there (e.g., Barber et al. 2006, Crandall et al. 2008, DeBoer et al. 2008, 2013a,b, Barber et al. 2011, Jackson et al. 2014). However, ecological factors that could lead to lineage diversification are difficult to identify because allopatric barriers in open marine environments are typically not distinct (Norris and Hull 2012). This is particularly true for the majority of marine species whose pelagic larval phase increases dispersal capabilities (Rocha and Bowen 2008).

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Despite presumptive widespread marine connectivity, phylogeographic studies suggest that physical boundaries have restricted gene flow between marine populations within the Coral Triangle (Barber et al. 2011, Carpenter et al. 2011). For example, a phylogeographic “break” between divergent mitochondrial clades is observed in a variety of species along the southwest side of the Sunda shelf, where genetically distinct populations of reef fishes and invertebrates have been traced to common ancestors that lived during the Pleistocene (McMillan and Palumbi 1995, DeBoer et al. 2008, Crandall et al. 2008, Gaither et al. 2011, Ackiss et al. 2013). During this time, repeated periods of extreme glaciation led to low sea levels and exposed the Sunda shelf. This exterminated all marine populations on the shelf and created an allopatric barrier between the Indian and Pacific oceans, resulting in genetically divergent populations of marine organisms. As sea levels rose, formerly isolated populations reunited. Some may have evolved into separate species, while in other cases, divergent populations remained as distinct demes of the same species (Benzie 1999).

Cycles of sea level fluctuation during the Pleistocene may also have had vicariant effects at smaller scales between basins within the Coral Triangle, contributing to the high interspecific and intraspecific diversity in the region (Carpenter and Springer 2005). Reduced gene flow between the South China Sea, Sulu Sea, and Celebes Sea, for example, appears likely when considering basin isolation from sea level reductions of around 120 m below what they are today (Fairbanks 1989; Fig. 1).

In addition to lineage diversification from sea level fluctuations, oceanographic features that have persisted over long periods may also have caused barriers to gene flow. Barber et al. (2002, 2006, 2011) suggested that the Halmahera Eddy entrains pelagic larvae and prevents their dispersal westward from Papua. The rabbitfish, *Siganus fuscescens* (Houttuyn, 1782), shows a population break, apparently as a result of limited dispersal across an oceanographic barrier at the Northern Equatorial Current Bifurcation (NECB) in the eastern Philippines (Magsino et al. 2008). Population breaks have also been observed in the giant boring clam, *Tridacna crocea* (Lamarck, 1819), in concordance with the Halmahera Eddy and the NECB (Ravago-Gotanco et al. 2007, DeBoer et al. 2008, 2014), and other giant clam species also show evidence for these oceanographic barriers, as well as other regions of genetic structure in the Coral Triangle not yet identified with oceanographic features (DeBoer et al. 2014). It is difficult to determine whether oceanographic currents serve as allopatric barriers that caused divergence or merely restrict the mixing of previously diverged populations, but it is apparent that they potentially provide a mechanism for isolation and speciation and influence population genetic structure of marine organisms. Allopatric barriers can lead to a build-up of genetic differences that eventually present intrinsic barriers to reproduction resulting in subsequent speciation (Dobzhansky 1970), although the strength of these barriers in the marine realm is often difficult to define (Palumbi 1994).

Carpenter et al. (2011) predicted that oceanographic features in addition to the Halmahera Eddy and Northern Equatorial Current Bifurcation might be uncovered within the Coral Triangle. Kool et al. (2010) made similar predictions using a biophysical model of larvae transported on ocean currents to infer population connectivity in the Coral Triangle. The present study suggested that the central Coral Triangle is a likely sink for pelagic larvae originating in genetically divergent populations in the Pacific Ocean and the South China Sea. Both Carpenter et al. (2011) and Kool et al. (2010) suggest that further studies including more extensive sampling

Figure 1. The central Coral Triangle. Sampling sites are marked by black dots and numbered. Pies represent relative abundance of clade 1 (white), clade 2 (black), and clade 3 (gray) based on Control Region neighbor-joining analysis and are sized to indicate relative sample size. Gray areas seaward of island boundaries indicate the 150 m depth contour.

are needed to establish an understanding of how ocean currents similar to those observed by Barber et al. (2006) and Magsino et al. (2008) might consistently affect a variety of species.

One example of how oceanographic currents could greatly influence dispersal and connectivity is through stepping-stone migration. The stepping-stone model of gene flow describes situations where the geographic distance over which an individual migration event occurs is smaller than the distributional range as intermediate populations serve to bridge connectivity across the species' range (Kimura and Weiss 1964). As currents influence both the directionality and distance of stepping-stone

dispersal events (Crandall et al. 2012), we can hypothesize that population connectivity is likely to occur in a stepping-stone fashion following the path of ocean currents. Conversely, strong currents could also act as an isolating mechanism. However, testing this hypothesis can be problematic because it can be difficult to sort out the relative influences of historical and contemporary environments on the distribution of populations.

According to the stepping-stone model, populations in equilibrium between migration and genetic drift are expected to show a pattern of isolation-by-distance (IBD), where the relatively great distance across the species' geographic range compared to the smaller distance between "stepping stones" of migration prevents complete panmixia (Wright 1943, Kimura and Weiss 1964, Slatkin 1993). However, adjacent populations that are genetically structured due to some barrier to gene flow can be easily confused with those that are structured due to IBD (Meirmans 2012). A partial Mantel's test for correlation between genetic distance and geographic distance can be used to determine if contemporary forces are likely to influence observed genetic patterns by distinguishing between IBD and regional population structure caused by distinct barriers to gene flow (Meirmans 2012).

Dascyllus aruanus (Linnaeus, 1758) is a damselfish whose life history characteristics provide potential for testing if distinct genetic boundaries exist within the Coral Triangle, and if they persist in predictable locations of limited connectivity. It is an easily recognizable, widespread Indo-Pacific species found on coral reefs throughout the Coral Triangle. It lays demersal eggs and has a pelagic larval duration ranging about 16–28 d before recruiting to the shelter of branching corals in shallow reefs and lagoons, where it maintains a highly restricted home range (Sweetman 1988, Wellington and Victor 1989). Moderate to low population structure has been found in this species (Planes et al. 1993), and similar results are expected within the Coral Triangle. Here we examine patterns of population structure of *D. aruanus* populations across the Philippine, Malaysian, and Indonesian archipelagos. Specifically, we test the hypothesis that populations from ocean basins that were potentially isolated during the Pleistocene will show evidence of genetic divergence and that contemporary oceanographic features will also structure these populations.

METHODS

Scuba divers collected a total of 401 *D. aruanus* individuals from 18 locations across the Philippines, Malaysia, and Indonesia (Fig. 1, Table 1) and preserved tissue samples in 95% ethanol. To avoid unnecessary damage to branching corals that make up adult *D. aruanus* habitat, collection tools consisted of hand nets and microspears made of sharpened umbrella spokes, ballpoint-pen shells, and rubber bands (A Bucol, Negros Oriental State University, pers comm). Several individuals of the sister species, *Dascyllus melanurus* (Bleeker, 1854), were also collected and kept for genetic comparison. The authors completed the laboratory work primarily in the respective countries where the tissues were collected (e.g., University of the Philippines Marine Science Institute in Quezon City and Universitas Udayana in Bali) or at Old Dominion University with permission from the host country.

We extracted genomic DNA with 10% Chelex (Walsh et al. 1991) or Qiagen DNeasy tissue extraction kits (Valencia, CA) according to the manufacturer's instructions, and amplified a 359-bp region of mitochondrial D-loop using the species-specific

Table 1. Sampling size (n), haplotype diversity, nucleotide diversity and Fu's F_s for Control Region and RAG2 data from each collection site (1–18). Location number refers to Figure 1.

Site	Control Region				RAG2			
	n	Haplotype diversity	Nucleotide diversity	Fu's F_s	n	Haplotype diversity	Nucleotide diversity	Fu's F_s
Philippines								
1. Romblon	36	0.9730	0.0297	-12.8517	N/A	N/A	N/A	N/A
2. Sorsogon	21	0.9667	0.0449	-0.6060	5(10)	1.0000	0.0103	-5.3943
3. Palawan	12	0.9091	0.0202	-0.1056	2(4)	1.0000	0.0075	-0.7685
4. Guimeras	10	0.9788	0.0205	-2.4318	N/A	N/A	N/A	N/A
5. Olango	42	0.9861	0.0238	1.9681	N/A	N/A	N/A	N/A
6. Siquijor	20	0.9526	0.0327	-2.1470	12(24)	0.9928	0.0135	-13.9886
7. Camiguin	18	0.9739	0.0199	-5.7005	N/A	N/A	N/A	N/A
8. Dinagat	20	0.9842	0.0385	-3.7120	2(4)	1.0000	0.0151	0.1179
9. Tawi-tawi	46	0.9159	0.0189	-10.7501	10(20)	0.9842	0.0121	-8.0689
10. General Santos	16	0.9667	0.0345	-1.8793	3(6)	0.9333	0.0041	-1.8459
11. Davao	13	0.8974	0.0429	2.4314	2(4)	1.0000	0.0112	-0.2530
Malaysia								
12. Sabah	23	0.9012	0.0192	-2.4162	N/A	N/A	N/A	N/A
Indonesia								
13. Manado	16	0.9333	0.0473	1.9681	N/A	N/A	N/A	N/A
14. Togians	18	0.8954	0.0224	-1.4759	N/A	N/A	N/A	N/A
15. Wakatobi	17	0.8824	0.0190	-0.5437	11(22)	0.0965	0.0056	-11.1434
16. Karimunjawa	19	1.0000	0.0321	-2.4318	N/A	N/A	N/A	N/A
17. Gilis	21	0.9619	0.0246	-4.0757	N/A	N/A	N/A	N/A
18. Komodo	33	0.9621	0.0452	-1.3713	10(20)	0.9947	0.0142	-11.4256
Total	401				57(114)			

primers CR-A DA (5'-ATG AAT CTT ACA ACT CAA CAC CTG-3') and CR-E DA (5'-TCA ACC AAG TAC AAC CCC TGT-3') (C Fauvelot, Institut de Recherche pour le Développement, pers comm) or the more general CR-A and CR-E (Lee et al. 1995). Polymerase chain reactions (PCR) were performed in 25- μ l reactions with 2.5 μ l of 10 $\times$ buffer, 2.5 μ l dNTPs (8 mm), 2 μ l MgCl₂ (25 mm), and 1.25 μ l of each 10 μ M primer, 1 μ l of template DNA, and 0.625 U of AmpliTaq (Applied Biosystems). Thermal cycler parameters included initial denaturation at 95 °C for 5 min, 38 cycles of 30 s at 95 °C, 30 s at 50 °C, and 40 s at 72 °C, and a final extension at 72 °C for 10 min. A 553-bp region of the nuclear recombination activation gene (RAG2) was also amplified and sequenced for a subset of 57 of our samples (Table 1) to determine if genetic patterns are consistent in the mitochondrial and nuclear genomes. These samples were a subset of the mitochondrial data set chosen to encompass divergent groups identified by analysis of that data (see results). We used the primers RAG2 F1 (5'-GAG GGC CAT CTC CTT CTC CAA-3') and RAG2 R2 (5'-GTC TGT AGA GTC TCA CAG GAG AGC A-3') (Cooper et al. 2009) and thermal cycler parameters consisted of denaturation at 95 °C for 5 min, 38 cycles of 30 s at 94 °C, 60 s at 54 °C, and 90 s at 72 °C, and a final extension at 72 °C for 7 min.

PCR products were cleaned by adding 0.5 units of Shrimp Alkaline Phosphatase and 5U of Exonuclease to 5 μ l of PCR product, and incubated at 37 °C for 30 min and 80 °C for 20 min. Forward and reverse sequencing reactions were performed with Big Dye 3.1 terminator chemistry (Applied Biosystems) and thermal cycler parameters

including 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 60 °C for 4 min. Cycle-sequenced products were then visualized at the Cornell University Life Sciences Core Laboratories Center on an ABI 3730xl automated DNA Sequencer (Applied Biosystems). Sequences were aligned and edited in Sequencher 4.10 (Gene Codes Corporation, Ann Arbor, Michigan). RAG2 sequences were phased using the Phase 2.1.1 algorithm (Stephens et al. 2001, Stephens and Scheet 2005) as implemented in the DnaSP 5 software (Librado and Rozas 2009). We ran five replicate runs with the default model, which allows for recombination, with each replicate consisting of 100 burn-in iterations followed by 100 iterations after stationarity was reached. We checked for convergence in inferred haplotypes across replicates, and used appropriate ambiguity codes for sites whose phase was inferred with a posterior probability <0.7.

We first investigated phylogenetic patterns with neighbor-joining analysis of Control Region and RAG2 data based on uncorrected p-distance in PAUP* 4b10 (Swofford 2002). We constructed a tree and rooted it with two sequences from the sister species *D. melanurus*. We also created a tree based on Bayesian inference in MrBayes (Huelsenbeck and Ronquist 2001) using the Jukes Cantor model as specified by analysis in jModeltest 2.1.3 (Darriba et al. 2011). The Bayesian tree construction included 1,000,000 generations, a burn in of 250,000 and a sample frequency of 1000. We visualized genetic relationships using FigTree 1.2.2 (Rambaut 2009), including clades with posterior probability >0.5. A median joining haplotype network was created in Network software (Bandelt et al. 1999) and edited, along with the neighbor-joining tree, in Adobe Illustrator CS3 13.0.1 (2007).

We assessed population structure with analyses of molecular variance (AMOVA) and pairwise F_{ST} values calculated in the Arlequin 3.11 software package (Excoffier 2005). Bonferroni corrections were calculated by hand (Bonferroni 1936). Input files for Arlequin were created using the online software FaBox (Villesen 2007). Significance was determined by P values <0.05, based on 10,000 permutations of the data for all analyses of molecular variance. We initially calculated Φ_{ST} and then considered traditional F_{ST} values without an underlying distance matrix among haplotypes to avoid spurious results arising from the presence of three major phylogenetic clades (Bird et al. 2011). Positions of potential genetic barriers were initially identified using the BARRIER 2.2 software (Manni et al. 2004) to test the association between genetic and geographic distances between sampling sites based on the Control Region data set. This analysis included no a priori assumptions of geographic locations for potential genetic breaks, and we only considered BARRIER results that were concordant with statistically significant pairwise F_{ST} values.

We grouped sampling sites into $K = 2, 3, 4$, and 5 potential subpopulations (clusters) with boundaries based on the following a priori information: (1) results of our observed phylogenetic patterns and BARRIER analysis; (2) predictions of expected population origins based on biophysical dispersal models described by Kool et al. (2010); (3) population boundaries found among other species in previous population genetics studies within the Coral Triangle (see figs. 1 and 2 in Carpenter et al. 2011); and (4) major physical factors within the region that are likely to act as barriers to larval dispersal, such as oceanographic currents (Hu et al. 2000, Kuhnt et al. 2004, Han et al. 2009) and land masses. The clusters were analyzed with grouped AMOVA (Arlequin 3.11) to achieve the highest F_{CT} values for each K value (2–5). This analysis was repeated without samples from Karimunjawa (site #16), and again with only the

samples from the most expansive of the observed clades (clade 1) to eliminate the possibility of type one error due to significance being driven exclusively by uniqueness of one (or both) of the two rarer phylogenetic clades. Significant AMOVA tests were also repeated with the RAG2 data to test for consistency of genetic structure in the nuclear genome.

The clustering schemes that resulted in the highest F_{CT} values, along with a $K = 1$ scenario, were evaluated as competing hypotheses in a model selection framework using the “Partition Compare” function in Bayesian Analysis of Population Structure (BAPS) 5.3 software (Corander and Tang 2007, Corander et al. 2008). Nuclear and mitochondrial data were analyzed in a variety of configurations in this program to compare the posterior probabilities and likelihood of the pre-specified clusters and to aid in determining the number of genetic units (K). The BAPS analyses were performed with the nuclear data, with all mitochondrial data, mitochondrial data in absence of Karimunjawa, and with only the data from clade 1, once again, to eliminate the influence of the two divergent phylogenetic clades.

All samples, and then data sets lacking each of the smaller hypothesized subpopulations identified by the AMOVA/BAPS analyses, were tested for the correlation between genetic distance and geographic distance (over water) expected under IBD, and for significance of hierarchical clustering with partial Mantel’s tests implemented in the *vegan* package (Oksanen et al. 2009) in R 2.15.1 software (R Core Team 2012). These tests used the Control Region data to determine whether genetic structure was better explained by barriers to gene flow or by IBD by considering the relationship of the genetic distances (G) to one of two distance matrices: over-water distances (O) and population membership (P), while using the other matrix as a covariate ($G \sim O + P$ and $G \sim P + O$). By simultaneously considering both hypothetical sources of genetic structure, partial Mantel tests can identify which one explains more of the genetic variance in the data, as has been shown with simulations (Miermans 2012). Ten thousand permutations of G were calculated for all partial Mantel tests, and subpopulations indicated by BAPS/AMOVA analyses were used for P.

RESULTS

Approximately 359 bp of mitochondrial Control Region were sequenced from 401 *D. aruanus* individuals from 18 sampling locations (Fig. 1, Table 1). All sequences aligned without ambiguity. In total, 202 distinct haplotypes were observed, with 35 different haplotypes found in more than one individual. Six of these were exclusive to only one sampling site. Four haplotypes occurred in more than 10 individuals, three occurred in more than 23 individuals, and the most common haplotype comprised 65 individuals (found at 16 of 18 sampling sites). There were 112 variable sites and only four indels, one of which was common to three individuals collected from Tawi-Tawi, Romblon, and Camiguin in the Philippines, another was found in four individuals from Palawan, Philippines, and the Gilis, Indonesia, and the final two indels were only found in single individuals from Karimunjawa, Indonesia. Five-hundred-and-fifty-three bp of nuclear RAG 2 were sequenced from 57 specimens from nine locations chosen to represent the divergent demes (clade 1 and clade 2) brought to our attention by previous analysis of the Control Region data (Fig. 1, Table 1).

Our neighbor-joining and Bayesian trees based on mtDNA (Fig. 2, Online Figs. 1, 2) revealed three major phylogenetic clades. The neighbor-joining tree separated the

Figure 2. Neighbor-joining trees drawn from Control Region data (left) and RAG2 data (right). Branch lengths are based on p-distance and both trees are rooted with two *Dascyllus melanurus* individuals (not included in figure). Narrow bars immediately to the right of each tree represent the placement of each individual in the three mitochondrial clades: clade 1 is white, clade 2 is black, and clade 3 is gray. Wide bars, farther to the right of each tree represent the regions from which each sample was collected: Karimunjawa (dark gray), Sulu Sea (light gray), Lesser Sunda Islands (black), and the remaining sites (white). Clade 2 (black) and clade 3 (gray) are absent in our samples from the Sulu Sea region.

clades (97%–100% bootstrap support) by approximately 20 mutational steps each (approximately 6% sequence divergence). Clade 1 (white) was found in all sampling sites except for Karimunjawa and included 355 individuals (Fig. 1). Clade 2 (black) was restricted to the central and eastern Philippines (Davao Del Norte, Dinagat, General Santos, Romblon, and Sorsogon), northeastern Sulawesi (Manado), and Komodo, and included 32 individuals. Clade 3 included only the 19 Karimunjawa samples. We obtained an additional mitochondrial sequence for this species from the Glorieuses in the western Indian Ocean, which also fell out in clade 3 (C Fauvelot, Institut de Recherche pour le Developpement, unpubl data). The haplotype network (Fig. 3) indicates genetic similarity between individuals within each clade. Neighbor-joining analysis of the RAG 2 data (Fig. 2) did not identify any apparent geographic pattern,

Figure 3. Haplotype network of Control Region data. Character differences of >1 are quantified with Roman numerals. Relative number of individuals in each haplotype are indicated by pie sizes. Black wedges represent proportions of individuals from Lesser Sunda sampling sites, light gray from Sulu Sea sites, dark gray from Karimunjawa, and white wedges represent individuals from all other sites. The smaller clusters separated by at >20 character mutations represents clade 2 and clade 3 while the remainder represents clade 1.

and both mitochondrial clades represented in the nuclear-based tree appeared to be randomly positioned throughout.

Barrier analysis identified potential genetic boundaries extending across the Sulu Archipelago between Tawi-tawi and General Santos, and between the Lesser Sunda Islands and sites north and west of there (Fig. 4). Barrier also isolated Karimunjawa from other sites. Initial ungrouped AMOVA analysis (Tables 2–3) showed that *D. aruanus* populations are structured within the Coral Triangle, $\Phi_{ST} = 0.2168$ ($P < 0.0001$), $F_{ST} = 0.0156$ ($P < 0.0001$). Global F_{ST} values remained significant in the absence of the Karimunjawa clade, $F_{ST} = 0.0143$ ($P < 0.0002$), and when calculated from only clade 1, $F_{ST} = 0.0139$ ($P < 0.0005$). The pairwise F_{ST} table identified significant structure between 41 pairs of sites. Following Bonferroni correction, few of these values were significant, with the exception of comparisons between Karimunjawa and Davao and Karimunjawa and Manado (Online Table 1).

The clustering schemes that resulted in the highest F_{CT} values for all of the data and for clade 1 data, along with ungrouped AMOVAs and BAPS results, are summarized in Tables 2–3. Grouped AMOVA tests resulted in the highest significant F_{CT} values for groupings including a “Lesser Sunda” cluster (Komodo and Gilis), and when Karimunjawa was isolated from the remaining sites. Clustering schemes that included a “Sulu Sea” cluster (Palawan, Tawi-Tawi, and Sabah), or a “southeast Sulawesi” cluster (Togians and Wakatobi), were not as strongly supported by AMOVA, though they were statistically significant. Calculations on data sets with and without the

Table 2. K values, AMOVA results (Φ statistics with P values), BAPS log-likelihood values, and posterior (post.) probability for significant imposed clustering schemes of sampling sites of the complete Control Region data set. Numbers in parentheses refer to specific sampling sites as can be viewed in Figure 1.

K	Imposed clusters	df	var	Φ_{CT}	P value	Φ_{SC}	P value	Φ_{ST}	P value	Loglikelihood	Post. probability
1	None	N/A	N/A	N/A	N/A	N/A	N/A	0.2168	0.000	-226142.384	0
2	Karimunjawa (16) + Remaining	1	11.9835	0.6851	0.000	0.0520	0.000	0.7015	0.000	-223968.410	0
3	Karimunjawa (16) + Sunda (17, 18) + Remaining	2	3.5558	0.3834	0.040	0.0479	0.000	0.4224	0.000	-224537.451	0
3	Karimunjawa (16) + Sulu (3, 9, 12) + Remaining	2	2.8399	0.3418	0.037	0.0454	0.000	0.3717	0.000	-223831.825	1
4	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	3	2.0098	0.2690	0.045	0.0441	0.000	0.3013	0.000	-224088.314	0
5	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi + Remaining	4	1.7390	0.2418	0.025	0.0424	0.000	0.2740	0.000	-224843.822	0

minority clades were consistent in supporting the same clusters for $K = 2, 3, 4$ or subpopulations. AMOVA analyses of RAG2 data alone did not result in any statistically significant structure among geographic groupings of sampling sites (Table 4).

Results of the BAPS analysis (Tables 2–3) indicated a value of $K = 3$ (1 = Karimunjawa, 2 = Sulu Sea, 3 = remaining sites) as the best fit when including all mitochondrial data (log likelihood = -223831.8248). In the absence of the minority clades, however, BAPS did not identify population structure. Clustering schemes that resulted in the highest F_{CT} values did not coincide with the highest likelihood values assigned by BAPS.

The partial Mantel’s tests (Table 5) indicated that population membership within clusters identified by AMOVA/BAPS analyses ($G \sim P + O$) is the primary correlate explaining genetic characteristics of the data rather than overwater distance ($G \sim O + P$). Significant r values signified structure at the Lesser Sunda Islands ($r = 0.3039, P < 0.05$) and the Sulu Sea ($r = 0.269, P < 0.05$) when all data were considered. Analysis of data in the absence of Karimunjawa also resulted in significant r values for presence of the Lesser Sunda Islands subpopulation ($r = 0.2486, P < 0.05$), and when considering only the white clade, partial Mantel’s test indicated that the genetic distance within that data set is best explained by a clustering scheme consisting of (1) Karimunjawa, (2) Sulu Sea, and (3) the remaining sites ($r = 0.08674, P < 0.05$), just as BAPS of all mitochondrial data indicates.

DISCUSSION

The pattern of deep phylogenetic divergence among *D. aruanus* populations and evidence of additional significant small-scale population structure corroborates previous studies that show a Sunda Shelf phylogeographic break (McMillan and Palumbi 1995, DeBoer et al. 2008, Crandall et al. 2008, Gaither et al. 2011, Ackiss et al. 2013) and suggests multiple causes of population structure in this species across the Coral Triangle. The separate major clade found exclusively at the Sunda

Table 3. K values, AMOVA results (F statistics with P values), BAPS log-likelihood values, and posterior (post.) probability for significant imposed clustering schemes of sampling sites of the complete Control Region data set, Control Region in absence of Karimunjawa (site 16), and the control region data including only clade 2. Numbers in parentheses refer to specific sampling sites as can be viewed in Figure 1.

K	Imposed clusters	df	var	F_{CT}	P value	F_{SC}	P value	F_{ST}	P value	Loglikelihood	Post. probability
All Data											
1	None	N/A	N/A	N/A	N/A	N/A	N/A	0.0156	0.000	-226,142.384	0
2	Karimunjawa (16) + Remaining	1	0.0071	0.0145	0.052	0.0142	0.000	0.0284	0.000	-223,968.410	0
3	Karimunjawa (16) + Sunda (17, 18) + Remaining	2	0.0059	0.0120	0.010	0.0116	0.002	0.0235	0.000	-224,537.451	0
3	Karimunjawa (16) + Sulu (3, 9, 12) + Remaining	2	0.0055	0.0114	0.008	0.0108	0.002	0.0220	0.000	-223,831.825	1
4	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	3	0.0050	0.0102	0.006	0.0095	0.009	0.0196	0.000	-224,088.314	0
5	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi + Remaining	4	0.0058	0.0120	0.001	0.0072	0.033	0.0191	0.000	-224,843.822	0
Without gray clade (Karimunjawa)											
1	None	N/A	N/A	N/A	N/A	N/A	N/A	0.0143	0.000	-197,665.696	1
2	Lesser Sunda (17, 18) + Remaining	1	0.0048	0.0100	0.024	0.0117	0.001	0.0216	0.000	-198,405.271	0
2	Sulu (3, 9, 12) + Remaining	1	0.0046	0.0094	0.028	0.0109	0.003	0.0204	0.000	-198,322.460	0
3	Lesser Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	2	0.0041	0.0085	0.016	0.0096	0.009	0.0180	0.000	-198,846.825	0
4	Lesser Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi (14, 15) + Remaining	3	0.0051	0.0105	0.004	0.0074	0.028	0.0178	0.000	-200,147.750	0
White clade only											
1	None	N/A	N/A	N/A	N/A	N/A	N/A	0.0139	0.000	-190,718.773	1
2	Lesser Sunda (17, 18) + Remaining	1	0.0059	0.0123	0.027	0.0109	0.007	0.0230	0.001	-191,920.732	0
2	Sulu (3, 9, 12) + Remaining	1	0.0032	0.0066	0.088	0.0114	0.005	0.0114	0.000	-191,003.908	0
3	Lesser Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	2	0.0037	0.0077	0.033	0.0096	0.018	0.0172	0.001	192,075.417	0
4	Lesser Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi (14, 15) + Remaining	3	0.0040	0.0084	0.018	0.0082	0.038	0.0166	0.000	-193,233.865	0

Table 4. K-values, AMOVA results (*F* statistics with *P* values), BAPS log-likelihood values, and posterior probability for imposed clustering schemes of sampling sites of the complete RAG2 data set. Numbers in parentheses refer to specific sampling sites as can be viewed in Figure 1.

K	Imposed clusters	df	var	<i>F</i> _{CT}	<i>P</i> value	<i>F</i> _{SC}	<i>P</i> value	<i>F</i> _{ST}	<i>P</i> value	Loglikelihood	Post. probability
1	None	N/A	N/A	N/A	N/A	N/A	N/A	0.0097	0.013	−87902.617	0
2	Lesser Sunda (16) + Remaining	1	−0.00170	−0.0132	0.551	0.0109	0.019	0.0075	0.013	−86026.442	0
2	Sulu (3,8) + Remaining	1	−0.00108	−0.0022	0.559	0.0105	0.016	0.0084	0.014	−89318.451	0
3	Sulu (3,8) + Lesser Sunda (16) + Remaining	2	−0.00230	−0.0046	0.707	0.0126	0.025	0.0081	0.013	−87489.200	0
4	Sulu (3,8) + Lesser Sunda (16) + SE Sulawesi (17) + Remaining	3	0.00440	0.0089	0.161	0.0022	0.371	0.0111	0.014	−81923.054	1

Shelf station (Karimunjawa) (Fig. 1) is most likely the one that dominates the Indian Ocean (C Fauvelot, Institut de Recherche pour le Développement, pers comm) and thus corroborates numerous other studies that have identified similar patterns of genetic structure in coral reef species across the region (DeBoer et al. 2008, Crandall et al. 2008, 2012, Gaither et al. 2011, Ackiss et al. 2013). It is worth noting that the mitochondrial break is more often found on the western side of the shelf rather than the eastern side as it is here (although see Barber et al. 2002a, 2006). This significant phylogeographic break supports the hypothesis that physical barriers, such as land barriers during Pleistocene low sea levels, once restricted gene flow between Indian Ocean and Pacific Ocean populations, and that this region represents an area where previously separated populations are coming into contact again.

While the distribution of deeply divergent clades highlights the importance of isolation across the Sunda Shelf in driving phylogeographic patterns in *D. aruanus*, the proximity of Indian Ocean haplotypes in Karimunjawa to highly divergent populations of the Lesser Sunda Islands suggests that more recent reproductive or physical barriers continue to limit genetic connectivity. However, with our data at hand, it is difficult to distinguish if an oceanographic feature such as the Indonesian Throughflow restricts larval dispersal between Java and the Lesser Sunda Islands following re-colonization of the Sunda Shelf by the Indian Ocean clade. Numerous other studies have shown genetic mixing between Sunda Shelf and Lesser Sunda populations (Barber et al. 2002a,b, 2006, Timm and Kochzius 2008, Ackiss et al. 2013) suggesting that the Indonesian Throughflow is not a barrier to larval dispersal for at least some species, and that ecological priority effects (Barber et al. 2002a) or reproductive barriers might also limit gene flow among *D. aruanus* populations of these two regions.

INFLUENCES OF ISOLATION ON LINEAGE DIVERSIFICATION.—Despite ambiguity regarding potential roles of oceanic and land boundaries across the area, our data also provide evidence to support the hypothesis that lineage diversification (potentially leading to speciation) originates within the Coral Triangle. In addition to the presumed allopatric barrier at the Sunda Shelf, Pleistocene glacial maxima lowered sea levels to around 120 m below present-day depths (Fairbanks 1989) and exposed other land barriers that may have led to isolation of populations within marine basins of the Coral Triangle (Voris 2000, Sathiamurthy and Voris 2006). Potential

for this can be seen when comparing estimated sea level minima to modern water depths throughout the region (Fig. 1). This may help to explain the genetic structure observed in *D. aruanus* at the Lesser Sunda Islands and in the Sulu Sea (Table 3). For example, Pleistocene low sea level stands might have led to reduced larval dispersal along the southern and eastern seaboard of the Philippine islands providing means for allopatric divergence at the shared borders of the Pacific Ocean, the Sulu and Celebes Seas, and the South China Sea. The presumably isolated, genetically divergent populations would only have come into contact with each other after sea levels rose above these boundaries. A barrier such as this, in transition from complete to partial, provides a potential explanation for the initial divergence and subsequent mixing of the phylogenetic clades observed in *D. aruanus* between the eastern and western Philippines (Figs. 1, 4). Taken alone, this population genetic structure could also be described as chaotic; however, when viewed in conjunction with phylogeographic results from other studies (Carpenter et al. 2011), a pattern potentially consistent with basin isolation emerges. For example, DeBoer et al. (2014) showed concordant patterns of population genetic structure across three species of giant clam somewhat similar to those observed in *D. aruanus*, particularly across the Sulu and Java Seas. In addition, Lourie et al. (2005) showed genetic structure across the Philippines in the seahorse, *Hippocampus spinosissimus* Weber, 1913, that is suggestive of similar basin isolation. These population genetic differences are not as trenchant as the consistent Sunda Shelf barrier between the Indian Ocean and Pacific Ocean basin, but the concordant patterns warrant further testing.

There are many ocean basins in the Philippines that were isolated during the glacial maxima (Voris 2000, Sathiamurthy and Voris 2006) and previous studies in the Coral Triangle have indicated population divergence among ocean basins associated with Pleistocene low sea level stands (Barber et al. 2000), so it could be predicted that *D. aruanus* should exhibit population structure corresponding to isolation within each of these basins. However, our data do not provide evidence of such a small-scale pattern of lineage diversification. Instead, the major pattern observed in the Philippines is differentiation in clade frequency between populations along the Pacific margin of the Philippines and those on the western side of the Sulu Sea. Connectivity between the Pacific Ocean and the western Sulu Sea might remain restricted by two major factors: (1) few narrow channels and restricted oceanic flow (Lermusiaux et al. 2011) that could inhibit passage of larvae east to west across the Philippine islands, and (2) the Sulu Sea Throughflow that could act as an east to west barrier across the Sulu Sea preventing larvae from crossing the flow of this persistent ocean current (Fig. 1). While the nearly continuous eastern seaboard of the Philippine Archipelago can continue to act as a filter, slowing and reducing the mixing of genetically distinct groups between the Pacific Ocean and the Sulu Sea, the Sulu Sea Throughflow might reinforce land barriers that once isolated populations more completely. The eastern Philippine barrier and the Sulu Sea Throughflow provide plausible explanations for the small scale divergence observed in our AMOVA results showing slight but significant F_{CT} values between Sulu sites and the remainder of the sampling locations (Table 3; all data and data without Karimunjawa). Additionally, Lourie et al. (2005) corroborated this pattern with evidence of highly divergent phylogeographic clade frequencies found on opposite sides of the Sulu Sea in three of four studied seahorses, in particular, *H. spinosissimus*.

Figure 4. Concordance among four complementary tests for population structure. Parallel dashed lines indicate analyses that identify potential regions of reduced genetic connectivity: (A) AMOVA, (B) BAPS, (C) partial Mantel tests, and (D) Barrier in concordance with significant pairwise F_{ST} values. Partial Mantel's tests were not used to test for uniqueness of the Karimunjawa group (16).

The idea that the Sulu Sea Throughflow and other contemporary oceanographic features also have the potential to restrict connectivity among *D. aruanus* populations across the region needs to be reinforced with additional quantitative support. Though there are some inconsistent results, concordance among significant F_{CT} and Φ_{CT} values in AMOVA (Tables 2–3), and partial Mantel's tests (Table 5) of multiple configurations of our data suggest that sites west of the Sulu Sea Throughflow, as well as the Lesser Sunda Islands, might represent independent clusters. Additionally, Kool et al. (2010) produced a current-based model of larval dispersal potential and concluded that the reefs of Sulawesi and Flores are expected to accumulate high levels

Table 5. Results of partial Mantel's tests comparing correlation (r) of geographic distance vs hierarchical clustering on F_{ST} for mitochondrial data sets including all data, and data the following samples removed (1) Karimunjawa, (2) Sulu Sea sites and Karimunjawa, (3) Lesser Sunda sites and Karimunjawa, and also data including the white clade only. Mantel models use the genetic data as the dependent variable explained by overwater distances (O) or population grouping (P). Names in parentheses represent the population grouping (separate Sunda or Sulu sites) that was used.

Data set	G ~ O + P (Sunda)		G ~ P + O (Sunda)		G ~ O + P (Sulu)		G ~ P + O (Sulu)	
	r	P	r	P	r	P	r	P
All data	-0.0805	0.799	0.3039	0.010	0.1618	0.087	0.2690	0.029
No Karimunjawa	-0.0393	0.062	0.2486	0.037	0.1851	0.066	0.2217	0.061
No Sulu or Karimunjawa	0.0633	0.275	0.1092	0.258	N/A	N/A	N/A	N/A
No Sunda or Karimunjawa	N/A	N/A	N/A	N/A	0.1657	0.127	0.1752	0.114
White clade only	-0.1039	0.832	0.2149	0.070	0.0572	0.326	0.0867	0.027

of diversity, which coincides with our observation of the highest levels of nucleotide diversity at Manado and Komodo. They went on to suggest that Palawan and the Sulu Archipelago should represent a distinguishable geographic region while the majority of larvae originating in the Sulu Sea remain there due to oceanographic features within the basin. Our data are consistent with these predicted areas of source and mixing. Two of the three *D. aruanus* clades we identified are observed in northern Sulawesi and eastern Flores where the highest mixing of stocks was predicted, as well as throughout the southern and eastern Philippines. Additionally, clade 2 genotypes are completely absent in the western Sulu Sea, an “upstream source of diversity” to the central Coral Triangle according to Kool et al. (2010).

With our present data it is difficult to distinguish if modern oceanic currents provide allopatric barriers that are responsible for the genetic divergence that we observe in *D. aruanus* or if the currents simply help to govern the interaction of previously diverged subpopulations. It is evident that oceanographic currents can act as a driving force of evolution of marine organisms in the Coral Triangle and that the pattern that we observe aligns, at least partially, with major oceanographic forces. Our partial Mantel tests indicate that a model of regional clustering might be helpful for describing population characteristics of *D. aruanus*, and possibly other marine species in the Coral Triangle, and they reiterate a need for further study on the specific roles of the Indonesian Throughflow and Sulu Sea currents on genetic connectivity of the species by specifically integrating predictive biophysical models and genetic data (e.g., Crandall et al 2012). Concordance across taxa including giant clams and seahorses suggests that similar oceanographic or historical factors might influence genetic structure for a variety of taxa within the Coral Triangle, while inconsistent phylogeographic observations among marine species continue to complicate our understanding of the relative roles of these factors on genetic connectivity in the Coral Triangle. Inconsistencies are expected because the large effective population sizes of Coral Triangle populations make it difficult to test for contemporary patterns of gene flow (Faurby and Barber 2012). Moreover, unique sampling locations, variance in life history cycles, and lottery effects in successful recruitment (Sale 1978, Marshall et al. 2010), along with cyclical changes in the flow of the major ocean currents and associated eddies at different time scales within the Coral Triangle (Han et al. 2009), may play a role in creating inconsistencies among taxa that experienced similar histories. Therefore, it is always important to note similar results found across taxa.

It is important to develop better understanding of population genetic characteristics of a great variety of marine species to better understand the processes of evolution in the Coral Triangle. Our study has corroborated numerous other studies that have identified population structure at the Sunda Shelf and has introduced other specific areas and potential sources of distinct populations within the Coral Triangle. Population structure observed across the Coral Triangle could be a result of Pleistocene ocean basin isolation, contemporary oceanographic features, or a combination of both. Analysis of larger data sets and more species can help to establish trends and confidence in the relative roles of past and present influences on the genetic structure of marine species within the Coral Triangle. We should continue to enhance cumulative data sets, analytical techniques, and theories that can help to explain how the Coral Triangle can best be subdivided based on patterns of genetic structure, how genetic barriers can be predicted, and how various factors can influence them. The end result will include a better understanding of evolution as a whole, as well as how to manage the world's vanishing biodiversity.

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