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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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BASIN ISOLATION AND OCEANOGRAPHIC FEATURES INFLUENCING LINEAGE DIVERGENCE IN THE HUMBUG DAMSELFISH (*DASCYLLUS ARUANUS*) IN THE CORAL TRIANGLE

by

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ABSTRACT

BASIN ISOLATION AND OCEANOGRAPHIC FEATURES INFLUENCING LINEAGE DIVERGENCE IN THE HUMBUG DAMSELFISH *(DASCYLLUS ARUANUS)* IN THE CORAL TRIANGLE

Jeremy M. Raynal
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The Coral Triangle (CT) is a hotspot for marine species diversity as well as for intraspecific genetic diversity. Here, nuclear RAG2 and mitochondrial D-Loop genes were used to identify deep genetic divergence among *Dascyllus aruanus* (Linnaeus, 1758) populations across relatively short scales within the CT. Mitochondrial clades different by greater than 20 mutational steps were geographically isolated from one another across the distance between Java and the Lesser Sunda Islands, and also east and west across the Philippines. Evidence for population structure in the Sulu Sea and at the Lesser Sunda Islands is also identified. The 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 are potential factors leading to lineage divergence of *D. aruanus*, and these hypotheses should be tested in further studies.

This thesis is dedicated to anyone who might benefit from my knowledge on this subject.

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CHAPTER I

INTRODUCTION

The Coral Triangle encompasses the greatest concentration of marine biodiversity on Earth (Stehli and Wells 1971; Rosen 1971; Sanciangco et al. 2013). This biogeographic pattern is typically hypothesized to result from species overlap, refuge, origin, or accumulation (Palumbi 1996; Carpenter et al. 2011). Isolation of populations and subsequent speciation within the Coral Triangle is integral to the origin hypothesis (Palumbi 1994). However, ecological factors that could lead to lineage diversification are difficult to identify because allopatric barriers in open marine environments are typically not distinct. This is particularly true for the majority of marine species that have a pelagic larval phase that increases dispersal capabilities.

Despite presumptive widespread marine connectivity, phylogeographic studies suggest that physical boundaries have restricted gene flow between marine populations within the Coral Triangle (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; Crandall et al. 2008; Gaither et al. 2011). During this time, repeated periods of extreme glaciation led to low sea levels and exposed the Sunda shelf (Lambeck et al. 2002). This exterminated or displaced 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 overlapped in range once again. 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 & 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).

In addition to lineage diversification from sea level fluctuations, oceanographic currents that have persisted over long periods may also have caused barriers to gene flow (Carpenter et al. 2011). 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) where the North Equatorial Current splits in the central eastern Philippines to flow both north and south from a single location (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). Other giant clam species also show evidence for these oceanographic barriers, as well providing evidence for other regions of genetic structure in the Coral Triangle not yet attributed to oceanographic features (DeBoer , Boston University, in review).

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 accumulation of genetic differences that eventually present intrinsic barriers to reproduction and 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 based initially on the limited number of studies available to provide concordant results. 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. This 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. There is evidence for this sink effect in the giant boring clam (*T. crocea*) that shows unidirectional gene flow into central Indonesia from northern and eastern populations (DeBoer , Boston University, in review). Both Carpenter et al. (2011) and Kool et al. (2010) suggest that further studies including more extensive sampling are needed in order 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.

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 & Weiss 1964). Oceanographic currents can greatly influence the distance between these migratory "stepping stones" for pelagic larvae. We can hypothesize that population connectivity is likely to occur along rather than across major currents, while population breaks should occur across them, simply based on larval dispersal probability (Kool et al. 2010). 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 (Kimura & Weiss 1964; Slatkin 1993; Wright 1943). 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 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 of approximately 16 to 28 days before recruiting to the shelter of branching corals in shallow reefs and lagoons where it maintains a highly restricted home range (Wellington & Victor 1989; Sweatman 1988). Moderate to low population structure has been found in this species (Planes et al. 1993), and similar results are expected within the Coral Triangle. The null hypothesis is that *Dascyllus aruanus* exists as a single panmictic population within the Coral Triangle. Alternatively, observed genetic structure is concordant with basins that were potentially isolated during the Pleistocene or contemporary oceanographic features.

CHAPTER II

METHODS

Scuba divers collected a total of 401 *D. aruanus* individuals from 18 locations across the Philippines, Malaysia, and Indonesia (Figure 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 pers comm.). Several individuals of the sister species, *Dascyllus melanurus* (Bleeker 1854) were also collected and kept for genetic comparison. The author completed the laboratory work at local universities in the respective countries where the tissues were collected.

Genomic DNA was extracted with 10% Chelex (Walsh et al. 1991) or Qiagen DNeasy tissue extraction kits (Valencia, CA, USA) according to the manufacturer's instructions, and amplified a 358-bp region of the mitochondrial D-loop gene using the species-specific primers CR-A DA (5' ATG AAT CTT ACA ACT CAA CAC CTG 3') and CR-E DA (5' TCA ACC AAGTAC AAC CCC TGT 3') (C. Fauvelot, pers. comm.) or the more general CR-A and CR-E (Lee et al. 1995). Polymerase chain reactions (PCR) were performed in 25 μL reactions with 2.5 μL of 10X buffer, 2.5 μL dNTPs (8 mm), 2 μL $MgCl_2$ (25 mm), 1.25 μL of each 10 μM primer, 1 μL of template DNA, and 0.625 U of AmpliTaq (Applied Biosystems). Thermal cycler parameters included initial denaturation at 95°C for 5 minutes, 38 cycles of 30 seconds at 95°C, 30 seconds at 50°C, and 40 seconds at 72°C, and a final extension at 72°C for 10 minutes. A 553-bp region of the nuclear recombination activation gene (RAG2) was also amplified and sequenced for

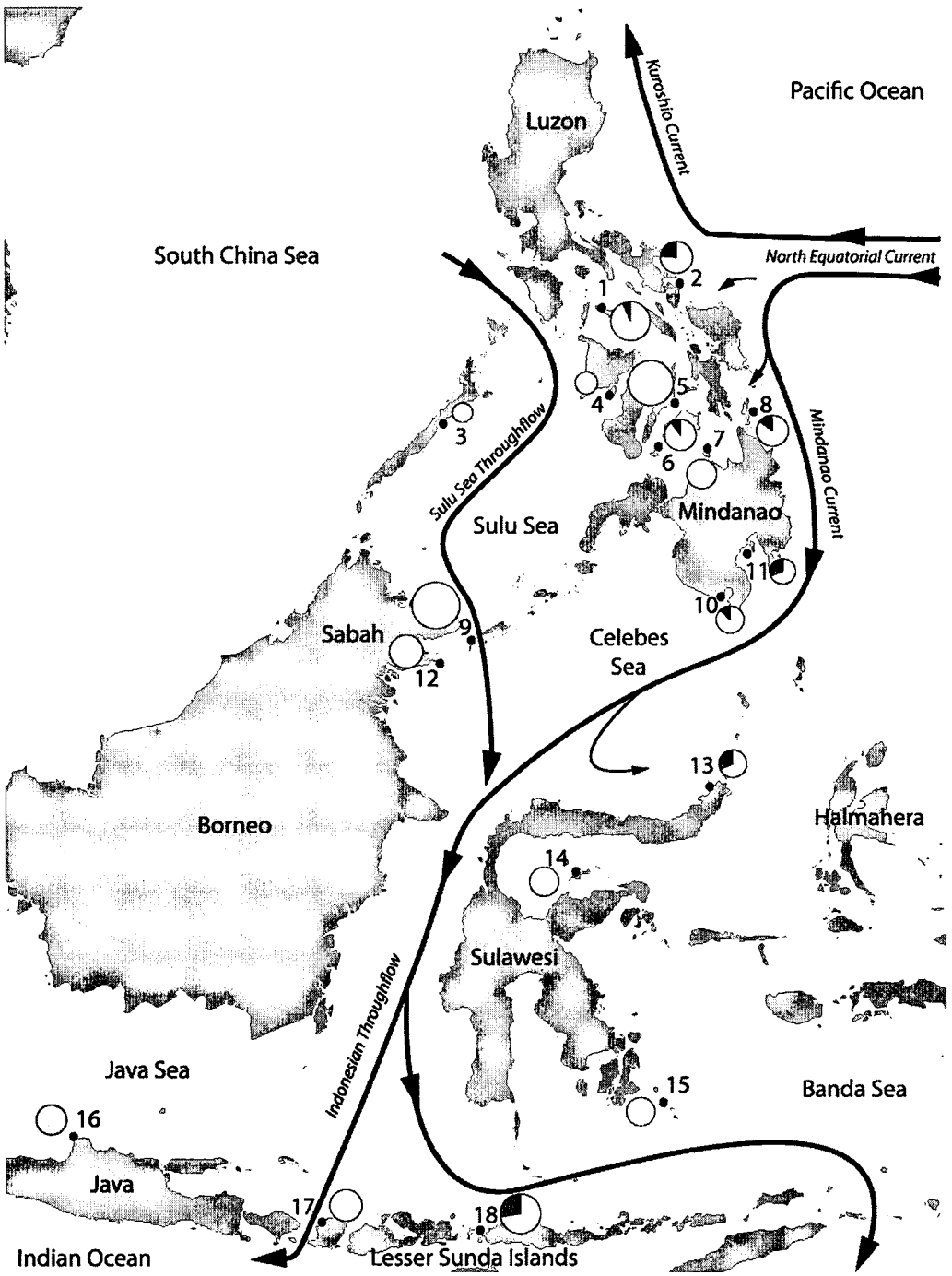

Figure 1. The central Coral Triangle. Sampling sites are marked by black dots and numbered. Pies represent relative abundance of Clade 1 (white), Clade 2 (black), and Clade 3 (grey) based on Control Region neighbor-joining analysis and are sized to indicate relative sample size. Grey areas seaward of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

Table 1. Sampling sites, haplotype diversity, nucleotide diversity, and Fu's F_S for Control Region and RAG2 data from each collection site (1-18). Location numbers refer to Figure 1.

	Control Region			
Site	***n (CR)***	***Haplotype diversity***	***Nucleotide diversity***	***Fu's Fs***
Philippines				
1 Romblon	36	0.9730	0.0297	-12.8517
2 Sorsogon	21	0.9667	0.0449	-0.6060
3 Palawan	12	0.9091	0.0202	-0.1056
4 Guimeras	10	0.9788	0.0205	-2.4318
5 Olango	42	0.9861	0.0238	1.9681
6 Siquijor	20	0.9526	0.0327	-2.1470
7 Camiguin	18	0.9739	0.0199	-5.7005
8 Dinagat	20	0.9842	0.0385	-3.7120
9 Tawi-tawi	46	0.9159	0.0189	-10.7501
10 Gereral Santos	16	0.9667	0.0345	-1.8793
11 Davao	13	0.8974	0.0429	2.4314
Malaysia				
12 Sabah	23	0.9012	0.0192	-2.4162
Indonesia				
13 Manado	16	0.9333	0.0473	1.9681
14 Togians	18	0.8954	0.0224	-1.4759
15 Wakatobi	17	0.8824	0.0190	-0.5437
16 Karimunjawa	19	1.0000	0.0321	-2.4318
17 Gilis	21	0.9619	0.0246	-4.0757
18 Komodo	33	0.9621	0.0452	-1.3713
Total	401			

Table 1. Continued.

	RAG2			
Site	*n (RAG2)*	*Haplotype diversity*	*Nucleotide diversity*	*Fu's Fs*
Philippines				
1 Romblon	N/A	N/A	N/A	N/A
2 Sorsogon	5(10)	1.0000	0.0103	-5.3943
3 Palawan	2(4)	1.0000	0.0075	-0.7685
4 Guimeras	N/A	N/A	N/A	N/A
5 Olango	N/A	N/A	N/A	N/A
6 Siquijor	12(24)	0.9928	0.0135	-13.9886
7 Camiguin	N/A	N/A	N/A	N/A
8 Dinagat	2(4)	1.0000	0.0151	0.1179
9 Tawi-tawi	10(20)	0.9842	0.0121	-8.0689
10 Gereral Santos	3(6)	0.9333	0.0041	-1.8459
11 Davao	2(4)	1.0000	0.0112	-0.2530
Malaysia				
12 Sabah	N/A	N/A	N/A	N/A
Indonesia				
13 Manado	N/A	N/A	N/A	N/A
14 Togians	N/A	N/A	N/A	N/A
15 Wakatobi	11(22)	0.0965	0.0056	-11.1434
16 Karimunjawa	N/A	N/A	N/A	N/A
17 Gilis	N/A	N/A	N/A	N/A
18 Komodo	10(20)	0.9947	0.0142	-11.4256
Total	57(114)			

a subset of 57 of the total samples (Table 1) in order to determine if genetic patterns were consistent in the mitochondrial and nuclear genomes. These samples for this work were chosen at random from each divergent group that was identified by analysis of the control region data (see results). 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) were used, and thermal cycler parameters consisted of denaturation at 95°C for 5 minutes, 38 cycles of 30 seconds at 94°C, 60 seconds at 54°C, and 90 seconds at 72°C, and a final extension at 72°C for 7 minutes.

PCR products were cleaned by adding 0.5 units of Shrimp Alkaline Phosphatase and 5U of Exonuclease to 5ul of PCR product, and incubated at 37°C for 30 minutes 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 seconds, 50°C for 30 seconds, and 60°C for 4 minutes. Cycle-sequenced products were then visualized by technicians at Cornell University Life Sciences Core Laboratories Center on an ABI 3730 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 & Scheet 2005) as implemented in the DnaSP v5 software (Librado & Rozas 2009). Five replicate runs were completed 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. A check for convergence in inferred haplotypes across replicates was completed, and appropriate

ambiguity codes were used for sites whose phase was inferred with a posterior probability < 0.7.

Phylogenetic patterns were first investigated with neighbor-joining analysis of control region and RAG2 data based on uncorrected p-distance in PAUP* 4b10 (Swofford 2002). A neighbor-joining tree was constructed and rooted with two sequences from the sister species *D. melanurus*. FigTree 1.2.2 (Rambaut 2009) was used to visualize genetic relationships. 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).

Population structure was assessed 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 of less than 0.05, based on 10,000 permutations of the data for all analyses of molecular variance. Φ_{ST} was initially calculated, and then traditional F_{ST} values were considered 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 v 2.2 software (Manni et al. 2004) to test the association between genetic and geographic distances between sampling sites. This analysis includes no *a priori* assumptions of geographic locations for potential genetic breaks and is based on statistically significant and non-significant pairwise F_{ST}-values. One hundred bootstrap replicates of the Control Region dataset were created according to Haddon (2001) and used in BARRIER.

Sampling sites were grouped into K = 2, 3, 4, and 5 potential subpopulations (clusters) with boundaries based on the following a priori information: 1) results of the 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 (Carpenter et al. 2011), 4) major physical factors within the region that are likely to act as barriers to larval dispersal, such as oceanographic currents (Kuhnt et al. 2004; Hu et al. 2000; Han et al. 2009) and land masses. The clusters were analyzed with grouped AMOVA (Arlequin 3.11) in order 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) in order to eliminate any possible influence of the two rarer phylogenetic clades on the grouping of sampling sites. Significant AMOVA tests were also repeated with the RAG2 data in order 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 & Tang, 2007; Corander et al. 2008). Nuclear and mitochondrial data were analyzed in a variety of configurations in this program in order 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, in order to eliminate the influence of the two divergent phylogenetic clades.

All samples, and then datasets lacking each of the smaller hypothesized subpopulations identified by the AMOVA/BAPS analysis 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 ~ O + P and G ~ 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.

CHAPTER III

RESULTS

Approximately 359 bp of mitochondrial control region were sequenced from 401 *D. aruanus* individuals from 18 sampling locations (Figure 1; Table 1). All sequences aligned without ambiguity. A total of 202 distinct haplotypes were observed, with thirty-five different haplotypes found in more than one individual. Six of these were exclusive to only one sampling site. Four haplotypes included more than 10 individuals, three included greater 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. 553 bp of nuclear RAG 2 were sequenced from 57 specimens from 9 locations chosen to represent the divergent demes (clade1 and clade 2) brought to our attention by previous analysis of the control region data (Figure 1; Table 1).

The neighbor-joining tree based on mtDNA (Figure 2) revealed three major phylogenetic clades (100% bootstrap support) separated by approximately twenty mutational steps each (approximately 6% sequence divergence). Clade 1 (white) was found in all sampling sites except for Karimunjawa and included 355 individuals (Figure 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

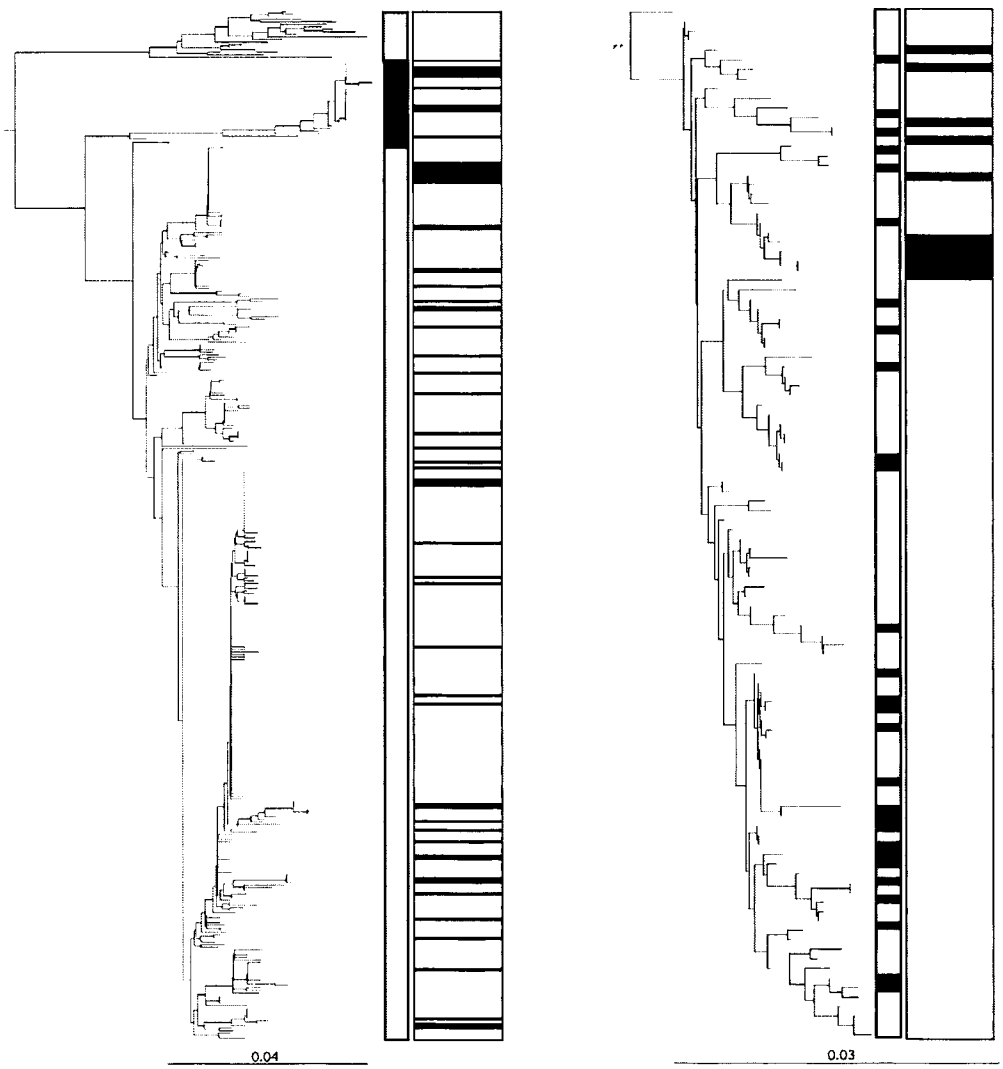

Figure 2. Neighbor-joining trees drawn from Control Region data (left) and RAG2 data (right). Branch lengths are based on p-distance and both trees are rooted with two *Dascyllus melanurus* individuals (not included in figure). Narrow bars immediately to the right of each tree represent the placement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

Komodo, and included 32 individuals. Clade 3 included only the 19 Karimunjawa samples, and also a single sample from Glorieuses in the western Indian Ocean (C. Fauvelot, Institut de Recherche pour le Developpement, unpublished data). The haplotype network (Figure 3) indicates genetic similarity between individuals within each clade. Neighbor-joining analysis of the RAG 2 data (Figure 2) did not identify any apparent geographic pattern, and both mitochondrial clades represented in the nuclear-based tree appeared to be randomly positioned throughout.

BARRIER analysis identified potential genetic boundaries extending in a complex fashion across the Sulu and Celebes seas, almost completely isolating the eastern Philippines and Manado from the western Philippines with greater than 80% bootstrap support (Figure 4). BARRIER also isolated Karimunjawa from other sites with very high confidence, and provided some evidence for further small-scale structure within the Philippines. Initial, ungrouped AMOVA analysis (Table 2-3), showed that *D. aruanus* populations are structured within the Coral Triangle, $\Phi_{ST} = .2168\ (p < 0.0001)$, $F_{ST} = 0.0156\ (p < 0.0001)$. 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. Bonferroni corrections produced insignificant values for previously observed pairwise structure, including values between sites that were characterized by highly divergent clades, with the exception of comparisons between Karimunjawa and Davao and Karimunjawa and Manado (S1).

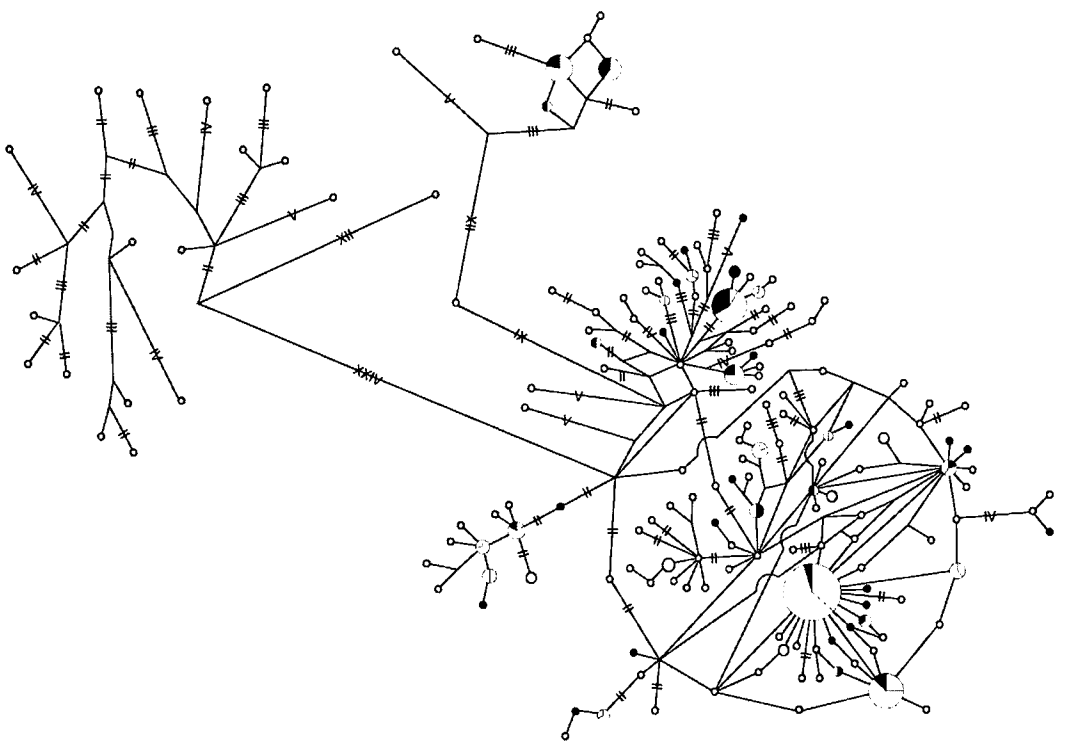

Figure 3. Haplotype network of Control Region data. Character differences of >1 are quantified with Roman numerals. Relative number of individuals in each haplotype are indicated by pie sizes. Black wedges represent proportions of individuals from Lesser Sunda sampling sites, light grey from Sulu Sea sites, dark grey 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.

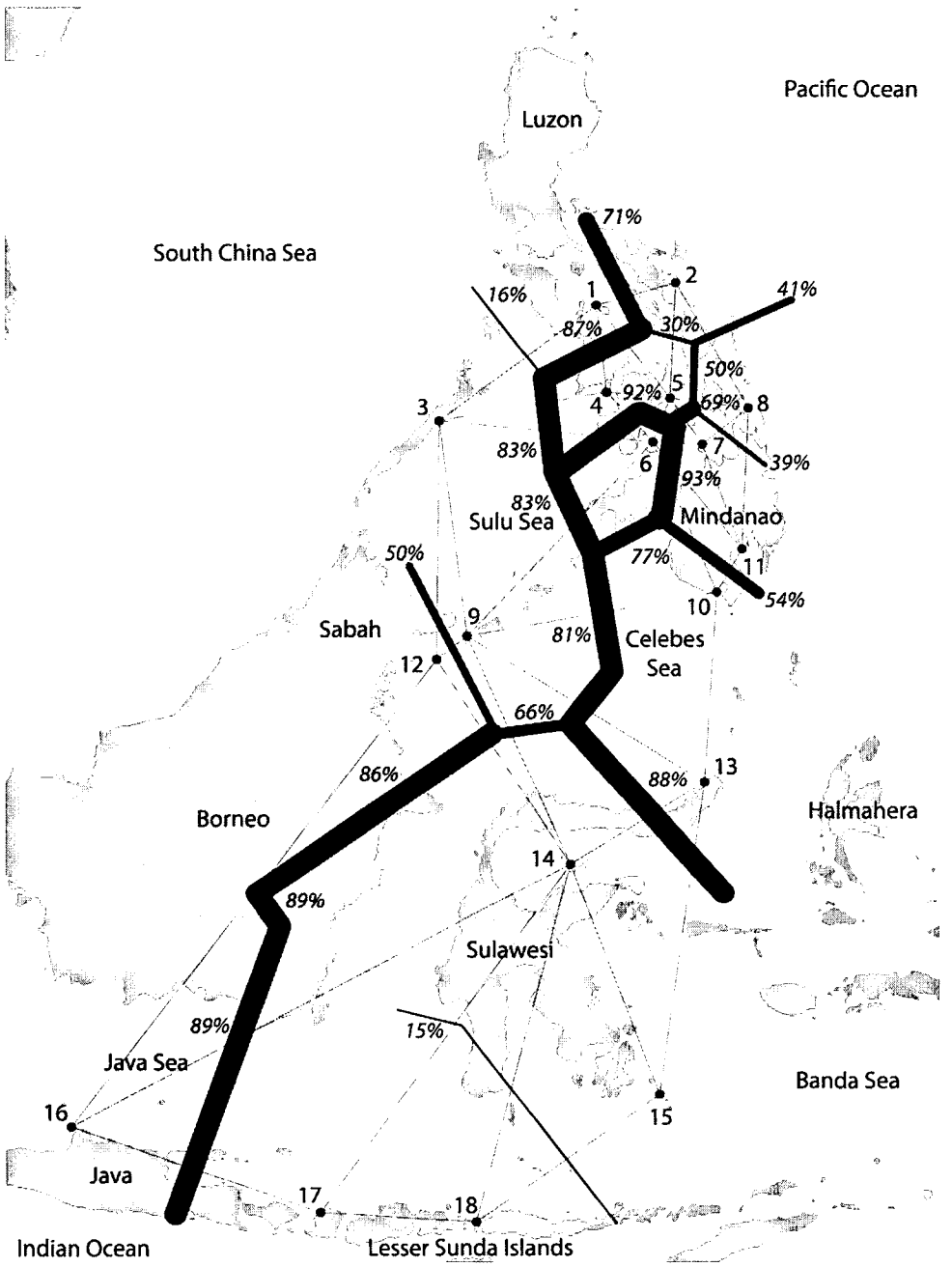

Figure 4. Results of the BARRIER analysis based on geographic and genetic distance (pairwise F_{ST}). Bars represent hypothetical regions of reduced genetic connectivity between neighboring sites, and their width indicates relative bootstrap support (also shown in %). The network of measured relationships are drawn between the numbered sampling sites.

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

K	*Imposed clusters*	*df*	*var*	Φ_{CT}	*p value*
1	None	N/A	N/A	N/A	N/A
2	Karimunjawa (16) + Remaining	1	11.9835	0.6851	0.000
3	Karimunjawa (16) + Sunda (17, 18) + Remaining	2	3.5558	0.3834	0.040
3	Karimunjawa (16) + Sulu (3, 9, 12) + Remaining	2	2.8399	0.3418	0.037
4	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	3	2.0098	0.2690	0.045
5	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi + Remaining	4	1.7390	0.2418	0.025

Table 2. Continued.

Φ_{SC}	*p value*	Φ_{ST}	*p value*	*loglikelihood*	*post. probability*
N/A	N/A	0.2168	0.000	-226142.384	0
0.0520	0.000	0.7015	0.000	-223968.410	0
0.0479	0.000	0.4224	0.000	-224537.451	0
0.0454	0.000	0.3717	0.000	-223831.825	1
0.0441	0.000	0.3013	0.000	-224088.314	0
0.0424	0.000	0.2740	0.000	-224843.822	0

Table 3. K-values, AMOVA results (F-statistics with p-values), BAPS log-likelihood, and posterior probability values for significant imposed clustering schemes of sampling sites of the complete Control Region dataset, 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.

Data set	*K*	*Imposed clusters*	*df*	*var*	F_{CT}	*p value*
All Data	1	None	N/A	N/A	N/A	N/A
	2	Karimunjawa (16) + Remaining	1	0.0071	0.0145	0.052
	3	Karimunjawa (16) + Sunda (17, 18) + Remaining	2	0.0059	0.0120	0.010
	3	Karimunjawa (16) + Sulu (3, 9, 12) + Remaining	2	0.0055	0.0114	0.008
	4	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + Remaining	3	0.0050	0.0102	0.006
	5	Karimunjawa (16) + Sunda (17, 18) + Sulu (3, 9, 12) + SE Sulawesi + Remaining	4	0.0058	0.0120	0.001
Without Grey Clade (Karimunjawa)	1	None	N/A	N/A	N/A	N/A
	2	Lesser Sunda (17, 18) + Remaining	1	0.0048	0.0100	0.024
	2	Sulu (3,9,12) + Remaining	1	0.0046	0.0094	0.028
	3	Lesser Sunda (17,18) + Sulu (3,9,12) + Remaining	2	0.0041	0.0085	0.016
	4	Lesser Sunda (17,18) + Sulu (3,9,12) + SE Sulawesi (14,15) + Remaining	3	0.0051	0.0105	0.004
White Clade Only	1	None	N/A	N/A	N/A	N/A
	2	Lesser Sunda (17, 18) + Remaining	1	0.0059	0.0123	0.027
	2	Sulu (3,9,12) + Remaining	1	0.0032	0.0066	0.088
	3	Lesser Sunda (17,18) + Sulu (3,9,12) + Remaining	2	0.0037	0.0077	0.033
	4	Lesser Sunda (17,18) + Sulu (3,9,12) + SE Sulawesi (14,15) + Remaining	3	0.0040	0.0084	0.018

Table 3. Continued.

F_{SC}	*p value*	F_{ST}	*p value*	*loglikelihood*	*post. probability*
N/A	N/A	0.0156	0.000	-226142.384	0
0.0142	0.000	0.0284	0.000	-223968.410	0
0.0116	0.002	0.0235	0.000	-224537.451	0
0.0108	0.002	0.0220	0.000	-223831.825	1
0.0095	0.009	0.0196	0.000	-224088.314	0
0.0072	0.033	0.0191	0.000	-224843.822	0
N/A	N/A	0.0143	0.000	-197665.696	1
0.0117	0.001	0.0216	0.000	-198405.271	0
0.0109	0.003	0.0204	0.000	-198322.460	0
0.0096	0.009	0.0180	0.000	-198846.825	0
0.0074	0.028	0.0178	0.000	-200147.750	0
N/A	N/A	0.0139	0.000	-190718.773	1
0.0109	0.007	0.0230	0.001	-191920.732	0
0.0114	0.005	0.0114	0.000	-191003.908	0
0.0096	0.018	0.0172	0.001	192075.417	0
0.0082	0.038	0.0166	0.000	-193233.865	0

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 minority clades were consistent in supporting the same clusters for K=2, 3, 4 or subpopulations. AMOVA analyses of RAG 2 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 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 ~ P + O) is the primary correlate explaining genetic characteristics of the data rather than overwater distance (G ~ O + P). Significant r-values signified structure at Lesser Sunda Islands (*r=0.3039, p=0.010299*) and the Sulu Sea (*r=0.269, p=0.028997*) when all data was considered. Analysis of data in the absence of Karimunjawa also resulted in significant r-values for presence of the

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

Data set	*K*	*Imposed clusters*	*df*	*var*	F_{CT}	*p value*
RAG2 Only (F)	1	None	N/A	N/A	N/A	N/A
	2	Lesser Sunda (16) + Remaining	1	-0.0017	-0.0132	0.551
	2	Sulu (3,8) + Remaining	1	-0.00108	-0.0022	0.559
	3	Sulu (3,8) + Lesser Sunda (16) + Remaining	2	-0.0023	-0.0046	0.707
	4	Sulu (3,8) + Lesser Sunda (16) + SE Sulawesi (17) Remaining	3	0.0044	0.0089	0.161

Tabel 4. Continued.

F_{SC}	*p value*	F_{ST}	*p value*	*loglikelihood*	*post. probability*
N/A	N/A	0.0097	0.013	-87902.617	0
0.0109	0.019	0.0075	0.013	-86026.442	0
0.0105	0.016	0.0084	0.014	-89318.451	0
0.0126	0.025	0.0081	0.013	-87489.200	0
0.0022	0.371	0.0111	0.014	-81923.054	1

Table 5. Results of partial Mantel's tests comparing correlation (r) of geographic distance versus hierarchical clustering on F_{ST} for mitochondrial datasets 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.

Mantel Model	**G ~ O + P (Sunda)**		**G ~ P + O (Sunda)**	
Dataset	***r***	***p***	***r***	***p***
All Data	-0.0805	0.799	0.3039	0.010
No Karimunjawa	-0.0393	0.062	0.2486	0.037
No Sulu or Karimunjawa	0.0633	0.275	0.1092	0.258
No Sunda or Karimunjawa	N/A	N/A	N/A	N/A
White Clade Only	-0.1039	0.832	0.2149	0.070

Table 5. Continued.

Mantel Model	G ~ O + P (Sulu)		G ~ P + O (Sulu)	
Dataset	***r***	***p***	***r***	***p***
All Data	0.1618	0.087	0.2690	0.029
No Karimunjawa	0.1851	0.066	0.2217	0.061
No Sulu or Karimunjawa	N/A	N/A	N/A	N/A
No Sunda or Karimunjawa	0.1657	0.127	0.1752	0.114
White Clade Only	0.0572	0.326	0.0867	0.027

Lesser Sunda Islands subpopulation (*r=0.2486, p=0.037296*), and when considering only the white clade, partial Mantel's test indicated that the genetic distance within that dataset is best explained by a clustering scheme consisting of 1) Karimunjawa, 2) Sulu Sea, and 3) the remaining sites (*r = 0.08674, p = 0.026667)*, just as BAPS of all mitochondrial data indicates.

CHAPTER IV

CONCLUSIONS

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 and suggests multiple causes of population structure. The separate major clade found exclusively at the Sunda Shelf station (Karimunjawa) (Figure 1), is most likely the one that dominates the Indian Ocean (C. Fauvelot pers. comm.) and thus corroborates numerous other studies that have identified similar patterns of genetic structure in coral reef species across the region (Carpenter et al. 2011), although generally the mitochondrial break is found on the western side of the shelf, rather than the eastern side as it is here (the given data provides no explanation for this). This significant phylogeographic break supports the hypothesis that physical barriers 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 (area of overlap hypothesis). The proximity of this major clade to highly divergent populations of the Lesser Sunda Islands suggests that more recent reproductive or physical barriers continue to limit genetic connectivity. However, with the 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, or if reproductive barriers inhibit gene flow between members of the grey clade and the other clades.

Despite ambiguity regarding potential roles of oceanic and land boundaries across the area, the data also provide evidence to support the hypothesis that genetic diversity (lineage diversification that potentially leads to speciation) originates within the Coral Triangle. In addition to the presumed allopatric barrier at the Sunda Shelf, Pleistocene glacial maxima lowered sea level 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. Potential for this can be seen when comparing estimated sea level minima to modern water depths throughout the region (Figure 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, prehistoric sea level lows 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 presumed 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 (Figure 1 & Figure 4). Taken alone, this population genetic structure could also be described as chaotic, however, when viewed in conjunction with phylogeographic results from other studies, a pattern potentially consistent with basin isolation emerges. For example, De Boer et al. (in review) show concordant patterns of population genetic structure across three species of giant clam surprisingly similar to those observed in *D. aruanus.* In addition, Lourie et al. (2005)

show 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.

Pleistocene barriers around the Coral Triangle potentially led to lineage diversification of populations, but present day oceanographic features also potentially influence gene flow between basins. Connectivity between the Pacific Ocean and the western Sulu Sea remains restricted by two major factors: 1) few narrow channels and restricted oceanic flow (Lermusiaux et al., 2011) that inhibits 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 (Figure 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. This could be a similar situation to the break between the Sunda Shelf and the Lesser Sunda islands that may be a result of the Indonesian Throughflow, possibly preventing east to west mixing of larvae by carrying them southward. The eastern Philippine barrier and the Sulu Sea Throughflow provide plausible explanations for the small scale divergence observed in the 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) corroborates 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*.

The idea that the Sulu Sea Throughflow and other contemporary oceanographic features restrict connectivity between *D. aruanus* populations across the region can be further supported by concordance of the AMOVA, partial Mantel tests (Table 5), and a previously published geophysical model (Kool et al. 2010). Though there are some inconsistent results, partial Mantel's tests of multiple configurations of the data indicate that sites west of the Sulu Sea Throughflow, as well as the Lesser Sunda Islands represent independent clusters of the sampling sites that are characterized by distinct haplotype frequencies, as detected in the grouped AMOVA. Additionally Kool et al. (2010) produced a current-based model of larval dispersal potential and concluded that the reefs of eastern Sulawesi, Flores, and the Philippines are expected to be among those that develop highest levels of genetic diversity. They went on to suggest that the Sulu archipelago is expected to act as the "connection point" between larval source regions of the South China Sea and the western Pacific, largely because of the strength and persistence of the Sulu Sea Throughflow. The presented data supports these predicted areas of source and mixing. Two of the three *D. aruanus* clades identified are observed in northern Sulawesi, eastern Flores, and throughout the eastern Philippines where the highest mixing of stocks was predicted, and Clade 2 genotypes are completely absent in the Sulu Sea, an "upstream source of diversity" to the central coral triangle according to Kool et al. (2010).

With the present data it is difficult to distinguish if modern oceanic currents provide allopatric barriers that are responsible for the genetic divergence that is observed

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 were observe aligns, at least partially, with major oceanographic forces. The 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 Throughflow currents on genetic connectivity of the species. Concordance across taxa including giant clams and seahorses suggest 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 make record of similar results found across taxa.

It is important to develop further understanding of population genetic characteristics of a great variety of marine species in order to better understand the

processes of evolution in the Coral Triangle. This 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 Philippines and at the Sunda Islands could be a result of Pleistocene ocean basin isolation, contemporary oceanographic features, or a combination of both. Analysis of larger datasets and more species can help to establish trends and confidence in relative roles of past and present influences on genetic structure of marine species within the Coral Triangle. We should continue to enhance cumulative datasets, analytical techniques and theories that can help to explain how the Coral Triangle can best be sub-divided based on patterns of genetic structure, how genetic barriers can be predicted, and how various factors can influence them. The end result will include better understanding of evolution as a whole, as well as how to manage the worlds vanishing biodiversity.

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VITA

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EDUCATION, CERTIFICATIONS, AND TRAINING

- B.S. (2001), Environmental Science (Biology), Chemistry Minor, University of North Carolina, Wilmington, NC
- AAUS Scientific Research Diver Certification and PADI Rescue Diver SCUBA license
- U.S. Coastguard Captain's License (Operator of Uninspected Passenger Vessels, 100 GRT)
- Grade 2 Wastewater Treatment Plant Certified Operator
- Coral Reef Ecology, Physical Oceanography University of the Philippines, Marine Science Institute (2007-2008)
- Virginia Saltwater Fishing Tournament Expert Saltwater Angler Award (2011)

PROFESSIONAL EXPERIENCE

- Old Dominion University, Graduate Teaching Assistant, Norfolk, VA, 8/10-12/12
- Coral Triangle Partnerships in International Research and Education, Norfolk, VA, 3/08-Present
- Global Marine Species Assessment, IUCN/ODURF, Norfolk, VA, 8/08-8/09
- U.S. Peace Corps, Coastal Resource Management, Bohol, Philippines, 3/05-6/07
- Super Soil Systems USA Inc., Agriculture Technology Development, Calypso, NC 7/02-5/04
- Enviromatters, Water Sampling, Testing and Treatment, Watha, NC, 1/02-5/02

PUBLICATIONS

Raynal JM, Crandall ED, Barber PB, Mahardika GM, Lagman MC, Carpenter KE. *In Review.* Basin isolation and oceanographic features influencing lineage divergence in the humbug damselfish (*Dascyllus aruanus*) in the Coral Triangle. Bull Mar Sci.

Raynal JM, Crandall ED, Barber PB, Mahardika GM, Lagman MC, Carpenter KE. *In Preparation.* Suture zones in the three-spot damselfish (*Dascyllus trimaculatus*) within the Coral Triangle.

PRESENTATIONS OF ORIGINAL RESEARCH

Raynal JM. 2012 Population genetics of humbug damselfish (*Dascyllus aruanus*). 41st Benthic Ecology Meeting, Norfolk Virginia.

Raynal JM. 2012. Population genetics of humbug damselfish (*Dascyllus aruanus*). Old Dominion University Biology Graduate Student Organization Spring Symposium, Norfolk, Virginia.