Genetic Connectivity of the Reef Building Coral *Pocillopora* sp. in the Red Sea

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1. Abstract

The Red Sea is a challenging environment for many marine organisms. It has one of the highest salinity concentrations (up to 40.2 ppt) and is the northernmost tropical sea worldwide. Due to almost complete isolation from the world's oceanic system it is additionally a very unique region in terms of biodiversity and a potential species incubator. The 2,000 km long (N/S) but narrow (W/E) basin, pushes strong latitudinal environmental changes especially regarding salinity (40.2–37.5 ppt) and temperature (28–33.8°C), which are the main stressors for most marine invertebrates and drivers of adaptive strategies. Nevertheless, its waters harbor a species rich and diverse environment, which is still widely unexplored. To assess the impact of the temperature and salinity gradient on the connectivity of reef organisms, the abundant reef-building coral *Pocillopora* sp. was sampled from North, Central and South offshore reefs. Ten out of fourteen microsatellite markers available for *Pocillopora* spp. were employed on individuals to detect the presence or absence of an underlying population genetic structure. To assure the exclusion of putative cryptic species, specimen used in this study were chosen from a single mitochondrial lineage. Samples were taken off the coast of Saudi Arabia and the sampled area covered over 850 km from North to South. Despite the long distances between collection sites, no significant genetic population structure was found, rejecting possible subdivision of the species driven by environmental factors and suggesting rather panmixia and sufficient gene flow (F_{ST} = 0.0005, *p = 0.340;* Variance among sites = 0.0%, based on 9 loci). This result is striking considering additional seasonal temperature fluxes from over 6°C and suggests high tolerance levels of *Pocillopora* sp. to diverse environmental conditions. Moreover, maintaining genetic diversity and frequent larval dispersal over wide distances and among contrasting habitats further supports the adaptive potential and reproductive success of *Pocillopora* species worldwide.

2. Introduction

2.1 The Red Sea

The Red Sea is an environment of extremes (Bethoux 1988, Sofianos *et al.* 2002, Smeed 2004, Ngugi *et al.* 2012). It is largely surrounded by desert but harbors one of the most species-rich habitats in the world, the coral reefs. Located between North Africa and the Arabian Peninsula it is the northernmost tropical sea worldwide. The flora and fauna living in these waters is not only living predominantly under extreme oligotrophic conditions (Sofianos *et al.* 2002) (characteristic for coral reefs) but is also exposed to extreme temperatures (over 33°C) and salinity contents (up to 41 psu) (Murray and Johns 1997; Sofianos and Johns 2007). The Red Sea basin is 2,000 km long and only few kilometers wide, equivalent to approximately one fifth of the Mediterranean Sea (Edwards 1987). The only connection to the open ocean is located in the South of the basin at Bab el Mandeb (Murray and Johns 1997), which is a very narrow and shallow strait to the Gulf of Aden (Peters and Johns 2005). Several physical conditions shape the climate of this tropical sea: (1) From North to South arid, dry and the hottest land regions influence surface water increasing its temperature to up to 33.8°C (in the South). Such temperature ranges combined with strong dry winds lead to high evaporation rates (Siddall *et al.* 2003; Sirocko 2003) of around two meters annually (Morcos, 1970). Regarding the lack of any potential fresh water source, the latter mentioned scenario explains top water temperatures and salinity concentrations (40.2 psu in the North) among the world's oceans (Edwards 1987; Trommer *et al.* 2009). (2) From South to North, less salty waters enter the Red Sea basin coming from the Gulf of Aden (Murray and Johns, 1997; Bower *et al.* 2000; Smeed 2004). As the waters of the North and the South start mixing salinity decreases proportionally to the amount of incoming waters from the Arabian Sea. In this manner an antagonistic temperature and salinity (T/S) gradient is build up following a North to South trend (24°C to 33.8°C and 41 psu to 36 psu respectively) (Edwards 1987, Trommer et a. 2009).

Habitat limitations of aquatic species are driven by parameters like temperature (Schwalbach *et al.* 2005; Zinser *et al.* 2007) and salinity (Hewson *et al.* 2006). Thus, the successful development of marine pelagic larvae is dependent on a certain range of these parameters defining the species habitat and shaping biogeography and the genetic

structure of the species population. In the Red Sea clear differentiation of populations along the latitudinal T/S gradient have been found for example among Crenarchaea (Trommer *et al.* 2009) and putative operational taxonomic units (OTUs) of Cyanobacteria and Proteobacteria, which were following a latitudinal biogeographic pattern (Ngugi *et al.* 2012). Furthermore, among other taxa, these extreme physicochemical conductions might be the result of endemism in the Red Sea (Weikert 1987, Roberts *et al.* 1992, Baars *et al.* 1998, Getahun 1998), speaking for the high influence of such in genetic differentiation (and even genetic drift).

2.2 The Study Case

In tropical oceans, some of the most delicate and biologically essential ecosystems are coral reefs. High biodiversity and productivity in oligotrophic waters are evidence to precise and unique productivity and sustainable use of limiting factors like nutrients (Sylvan *et al.* 2006). An effective usage and recycling of the enrolled ecosystems' nutrients can only be provided by strong and precise niche adaptation of the reef inhabitants entangled in delicate food webs (Garren and Azam 2012). A three-dimensional structure in the ecosystem is important to increase the space available for the niche establishment of thousands of species (Garren and Azam 2012) and is provided predominantly by stony corals. These use energy produced by symbiotic green algae to metabolize CO_2 and calcium to aragonite in order to build their skeleton on which this ecosystem relies on in so many ways (Muscatine and Cemichiari 1969; Johannes *et al.* 1970; Lewis and Smith 1971; Johannes and Tepley 1974; Porter 1974; Muscatine and Porter 1977). For the survival of corals and a successful calcification reaction optimal environmental conditions are indispensable. Therefore, the tolerance levels of physicochemical parameters of corals are very limited. Small differences of e.g. temperature (of only approx. 0.5-1°C) can already have lethal consequences. Additionally, most of the world's corals have their tolerance maximum around 30°C, which make corals in the Red Sea living above this temperature maximum increasingly fascinating.

In this study, we want to assess the influences of temperature and salinity on the reef-building coral *Pocillopora* sp. in the Red Sea. This is a common hard coral in most tropical reefs in the Indo-Pacific Ocean and the Red Sea. For this purpose, specimen

from North, Central and South located reefs along the west coast of Saudi Arbia (Fig. 1) are genetically analyzed employing formerly designed microsatellite markers (nuclear DNA) (Magalon *et al.* 2004; Starger *et al.* 2008). These are non-coding nuclear DNA (nDNA) loci, which due to the presence of short tandem repeats, lead to the occasional slippage of the DNA polymerase during DNA replication inducing the production of alleles with different number of repeats within the microsatellite loci. Such loci are, therefore, highly variable between individuals of the same species (i.e. have a high mutation rate) depending on their genealogical heritage and are hence, perfect for the analysis of recent genetic structuring and differentiation of populations. By utilizing these markers on *Pocillopora* sp. we expect to enlighten the population's structure and infer the influence of temperature and salinity on the species in the Red Sea. Furthermore, with population genetics, reproductive mode and extent of gene flow can be approximated and compared to results from other studies on connectivity (like e.g. Stoddart 1984, Johnson and Black 1984, Ayre *et al.* 1991, Ayre *et al.* 1997; Ayre *et al.* 2000). This reveals the state and resilience of the species as well as potential sources of the population's genetic diversity (Ayre and Dufty 1994; Benzie *et al.* 1995).

Seasonal variations in temperature have showed effects on reproduction of many marine invertebrates (Giese and Pearse 1974) and among these also of several coral species (Kojis and Quinn 1981, 1982; Stoddart and Black 1985; Babcock *et al.* 1986a, b). For example sea temperature in the Red Sea is said to be the controlling force for the onset of gametogenesis in *Pocillopora verrucosa* (Fadlallah 1985). Hence, we hypothesize that the T/S gradient in the Red Sea is significantly influencing the population structure of *Pocillopora* sp. and leading to genetic differentiation according to the geographic site and its characteristic physicochemical conditions. If latter hypothesis holds true, panmixia among the sampling sites in this study could be rejected.

3. Materials and Methods

3.1 Sampling

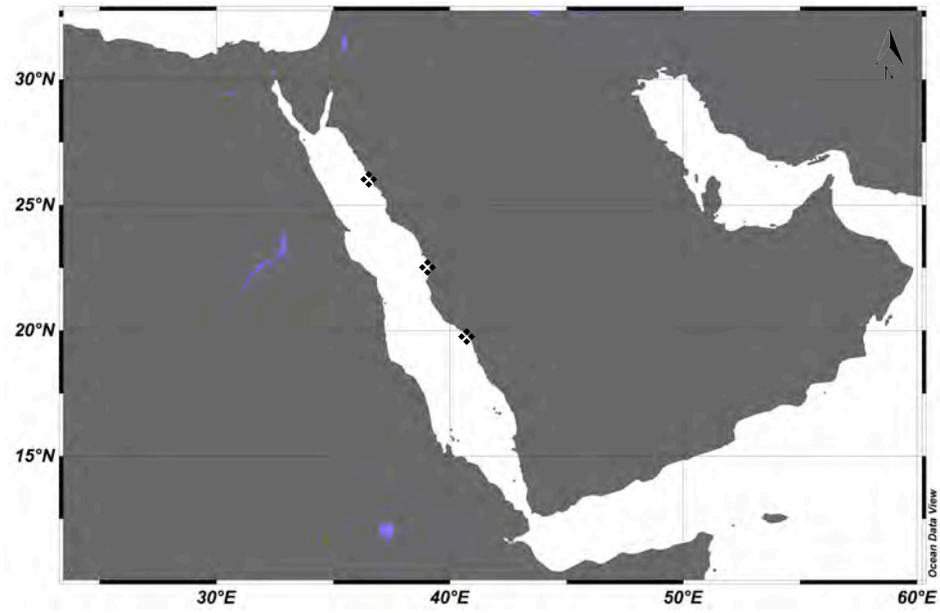

Fig. 1: Map of the Red Sea: Sampling sites of *Pocillopora* sp. are indicated with a star (❖). From North to South the indicated geographic locations are WAJ, KAU and DOG. Oceanic waters are *white*, Land is colored *gray* and land waters in *blue*.

Genetic data of the hard coral *Pocillopora* sp. was generated from samples of North, Central and South located offshore reefs off Saudi Arabia to evaluate gene flow and the population's structure in the Red Sea (Fig 1.). The map in Fig. 1 was generated with Ocean Data View 4.5.3 (ODV)

Samples of 1 to10 cm in length were collected from branches of the morphotype *Pocillopora verrucosa* (Fig. 2) in 2011, 2012 by Dr. Yvonne Sawall (GEOMAR) and Dr. Abdulmohsin Sofyani (KAU), and in 2013 by Vanessa Robitzch (KAUST). These were fixed in salt saturated DMSO solution and stored at -20 °C. Collection sites were *Al Waj* (WAJ) in the North, *Thuwal* (KAU) centrally located, and *Doga* (DOG) in the South. Three (KAU; DOG) to four (WAJ) offshore reefs were randomly sampled per location with at least 5 meters distance between each sampled colony to avoid sampling clones. Sampled reefs and number of samples per site were: off *Al Waj* (WAJ) - 44 samples from reef number one (R1), and 30 samples from each R2, R3 and R4; off *Thuwal* (KAU) - 69 samples (R1), 63 samples (R2) and 54 samples (R3); and off *Doga* (DOG) - 30 samples (R1 and R3) and 27 samples (R2) (see Tab. 1). Photographic

vouchers of the samples were also taken in the 2011 and 2012 sampling cruises.

For the amplification of the mitochondrial putative control region *igr11* (*CR*), the open reading frame (*ORF*) as well as nuclear microsatellites (MSATs), coral DNA was extracted from collected samples as follows.

Fig. 2: Two different morphotypes of collected *Pocillopora* sp. in the Red Sea (KAU) (copyright: Vanessa Robitzch).

3.2 DNA Extraction

DNA from all samples of DOG and WAJ plus 48 samples per reef of KAU (Tab. 1) was extracted following the manufacturer's protocol of the Qiagen DNeasy Plant Mini Kit. Here fore, smaller pieces of the coral samples (approx. 5mm diameter) were cut off with sterile forceps (to avoid cross contamination) and placed in a sterile 1.5 ml tube filled with approx. 0.5 ml of 0.5 mm glass beads (from Biospecs) and 400 µl AP1 Qiagen Lysis Buffer. To remove the tissue from the coral skeleton The Qiagen Tissue Lyser II was used at highest frequency (30 Hz) for 90 seconds. For the last step of the protocol, DNA elution was performed only once with 80 µl instead of 100 µl Qiagen AE elution buffer.

DNA content of the extraction was measured using the Thermo Scientific NanoDrop 2000 and DNA was diluted with Teknova DNase/RNase free PCR Water to a DNA concentration of approx. 10-40 ng/µl. Same diluted aliquots were used in the analyses of sections 3.3 and 3.4.

Tab. 1: Sampling Sites and Number of Samples for Mitochondrial (mtDNA) and Nuclear DNA (nDNA) Analyses

Site	Reef	N	N_{mt}	HP_A	HP_B	HP_C	HP_D	HP_E	HP_F	N_{MSAT}
(*WAJ*)	**R1**	44 (134)	44 (134)	12(20)	7 (23)	3 (9)	0 (5)	10(32)	12(43)	32 (89)
	R2	29	29	2	6	2	2	7	10	19
	R3	29	29	3	4	3	2	10	7	22
	R4	30	30	3	6	1	1	5	14	16
(*KAU*)	**R1**	69 (186)	48 (144)	9 (25)	5 (13)	1 (5)	1 (13)	17(43)	15(45)	33 (99)
	R2	63	48	7	3	2	6	13	17	31
	R3	54	48	9	5	2	6	13	13	35
(*DOG*)	**R1**	30 (87)	30 (87)	4 (14)	5 (13)	3 (7)	1 (9)	9 (21)	7 (21)	22 (64)
	R2	27	27	4	4	2	3	5	9	18
	R3	30	30	6	4	2	5	7	5	24
Total			*365*	*59*	*49*	*21*	*21*	*96*	*109*	*252*

Site: geographic sampling site (see also Fig. 1); **Reef**: Number of reefs per site; **N**: Number of samples per reef and per site in *parenthesis*; $\mathbf{N_{mt}}$: number of samples used for the mtDNA analysis. $\mathbf{HP_{A-F}}$: Number of samples per mitochondrial haplotype. $\mathbf{N_{MSAT}}$**:** Number of samples used for population genetics (after exclusion of the HP_F).

3.3 Mitochondrial DNA Markers – Preliminary Analysis

3.3.1 *igr11* - Putative Control Region (*CR*)

3.3.1.1 Amplification and Sequencing

As a first step in the analysis, a subset of 83 randomly picked samples from all four WAJ reefs was analyzed for variation of the mtDNA putative control region (*CR*).

Amplification of a fragment around 1,250 base pairs (bp) was initially carried out in 12 µl reactions containing 6 µl Qiagen Multiplex PCR Kit, 1.25 µM (0.125 µl of 10 µmol/l primer) of each primer: FNAD5.2deg *5'-GCCYAGRGGTGTTGTTCAAT-3'* and RCOI3 *5'-CGCAGAAAGCTCCAATCGTA-3'* (Flot *et al.* 2008), 3.75 µl Teknova DNase/RNase free PCR Water and 1 µl DNA (10-40 ng) as template. PCR conditions were established on an Eppendorf Thermocycler as follows: 94°C (15min), 35 cycles of 94°C (30s) denaturation, 53°C (45s) annealing and 72°C (90s) elongation followed by final elongation at 72°C (10min). This fragment was bi-directionally sequenced with the same primers by Flot *et al.* (2008). For a better quality and longer sequences, PCR products were re-sequenced in both directions using a different primer pair Rs-F15 *5'-G CGGGATCTTTAATCCATGC-3*' and Rs-R15 *5'-AAGCATACTAAAAGCAGTCC-3'* (Lin *et al.* 2011).

All PCR products were cleaned with QIAGEN ExoSAP before sequencing

following the manufacturer's protocol (2 µl ExoSAP for 5 µl PCR product). Sequencing was always performed on an ABI-3730xl Analyzer using the ABI Big Dye 3.1 Kit and ABI-Terminator Beads for clean up. The same machine was used for the fragment analysis under section 3.4.2.

3.3.1.2 Data Analysis

Sequences were assembled and analyzed using Code Aligner version 3.7.1 (CodonCode Corporation 2007-2009). Here fore, forward and reverse sequences were assembled separately and trimmed according to the sequence's quality. Each resulting contig was then manually examined for the presence of mutations.

3.3.2 *ORF* – Open Reading Frame

3.3.2.1 Amplification and Sequencing

The *ORF* of the mitochondria was amplified using the primers developed by Flot *et al.* (2008) *FATP6.1 5'-TTTGGGSATTCGTTTAGCAG-3'* and *RORF 5'-SCCAATATG TTAAACASCATGTCA-3'*. PCR reactions were set up in the same way as mentioned for *igr11*. The PCR cycle profile was: 94°C (15min), 35x [94°C (30s), 53°C (45s), 72°C (90s)] and 72°C (10min). Sequences were also generated as stated in 3.3.1.1.

3.3.1.2 Data Analysis

All sequences were aligned and trimmed in Codon Code Aligner version 3.7.1 (Codon Code Corporation 2007-2009) and re-sequenced if the sequence's quality was low. Only high quality unambiguous sequences were used for this analysis. The trimmed sequences' contigs (consisting of forward and reverse sequence) were exported to MEGA 5.1 to double check for gaps, inserts, deletions and then imported to DnaSP 5.10.1 (Librado *et al.* 2009) to generate a haplotype data file in NEXUS format. This file was imported to MEGA 5.1 (Tamura *et al.* 2011) to create a phylogenetic tree for

the *ORF* mtDNA haplotypes and cluster putative mitochondrial lineages.

3.4 Microsatellite nuclear DNA (nDNA) Markers – Population Genetics

3.4.1 Primer selection and Literature Consultation

Current literature was consulted to find suitable primers for the amplification of microsatellites (MSATs) and/or short tandem repeats (STRs) within the nuclear DNA of the *Pocillopora* genus. In total, 14 suitable primer pairs were initially tested (see Tab. 2). From each primer pair, the 5'-primer was labeled with red (AT565), yellow (AT550), green (HEX) or blue (FAM) fluorescent dyes (see Tab. 2). Microsatellite primers designed for the sister species *Stylophora* sp. (Banguera *et al.* 2013) were also tested on *Pocillopora* samples. However, none of the primer pairs for Stylophora sp. amplified desired MSAT regions.

Tab. 2: Microsatellite (MSAT) Primers for *Pocillopora* spp.

Name	Primer F+R (5'-3') (li.)	STR motif (li.)	N_a (li.)	MPLX No.	$T_{ann.}$ (ºC)	Frag. Length	Repeat (bp)	$N_a(N_{ap})$	Label
Pd1	CAGACTTGTCGGAATGAAAGC TTTTGTTTATAAGTCGATACAATGCA	$(CA)_{11}$	5	1	55ºC	194-206	2	6 (2)	5'-AT565
Pd2	ATCCGAATACAAGCGAAACG CAAAGCTTCTATCAGAAAATGCAA	$(AAC)_{10}$	10	1	55ºC	159-202	3	5	5'-AT550
Pd3	CCTCTTCCTGTTTGGGCTCT TCTGCATTACGTTTGTTTGACA	$(CA)_{16}$	3	/	59ºC	198-202 (li.)	2	/	5'-HEX
Pd4	ACCAGACAGAAACACGCACA GCAATGTGTAACAGAGGTGGAA	$(ATG)_8$	4	3	55ºC	156-180	3	8 (2)	5'-FAM
Pd5	AGAGTGTGGACAGCGAGGAT GTTCCTTCGCCTTCGATTTT	$(TGA)_9$	3	2	55ºC	189-237	3	15 (4)	5'-FAM
Pd6	ATCTCCATGTGATCGGCATT GTTCCCCCAGCTGAGAAGTT	$(CA)_8$	7	3	55ºC	186-296	2	13 (1)	5'-HEX
Pd7	AAGAAGGTGTGGTATTTCAGAGGG GGTGGATAAAGTATTTCTCACTCTTGG	$(AC)_{imperfect}$	7	4	50ºC	162-352	2	2 (6)	5'-AT565
Pd8	AGTTGAGGTTGTTGAAACATG TCCATGCAGAACCCC	(CTG)7	4	1	55ºC	161-185	3	9 (1)	5'-FAM
Pd9	CCAATGCGTCCGTAGCTCTC ATCACCTAAAAATTTCAGTCCCTTACC	(CAA)7,(GAG)6	6	4	50ºC	329-344	3	6 (0)	5'-HEX
Pd10	CTGATCAACAAACTGGGAGGC TCATTAGAAATCATCTTGATTTGATAAGG	(GTT)5, (TGC)11	7	/	/	259 -281 (li.)	3	/	/
PV2	GCCAGGACCCATTTATACTCC TGCAGTGTTCTACTTGTCAGTGC	(GA)20	7	2	55ºC	119-263	2	48	5'-HEX
PV5	GGTCATCACGCAAAGTTCC GAATAGCCTGCGTTTATTTGG	(CA)11	12	2	55ºC	231-239	2	5 (1)	5'-AT550
PV6	CTTTCCCGACCAGTTTAGGG AGCCGTTCAGCTACCTATGG	(GT)7	14	3	55ºC	203-221	2	10 (1)	5'-AT550
PV7	GGAGATGGATGGAGACTGC GGAGATGGATGGAGACTGC	(GT)5(CT)2GT(CT)3	5	1	55ºC	231-239	2	4 (0)	5'-AT565

Name: *Pd* -primers by Starger *et al.* (2008); *PV* -primers by Magalon *et al.* (2004)); **(li.):** information from literature; **STR**: Short Tandem Repeat; **N_a:** number of alleles; **MPLX No.**: Number assigned to the multiplex (MPLX) reaction; **$T_{ann.}$:** Annealing temperature of the primers after re-standardization; **Frag. Length:** Range of allele size/Fragment lengths; **N_a:** number of private alleles; **Label:** Fluorescent label used for the MSAT primer.

3.4.2 Amplification, Fragment Analysis and Genotyping

PCR conditions for each primer had to be re-standardized for the amplification of a total of 254 samples from one mitochondrial lineage based on the results from 3.3.1.2. PCR fragment lengths, quantity and quality was checked and estimated with the QIAxcel machine and the QIAxcel DNA High Resolution Kit. Once primers were amplifying the putative correct fragments, possible primer combinations for multiplex (MPLX) PCRs were tested and new PCR conditions were standardized for the primer set of the MPLX reactions.

13 total primer pairs were finally multiplexed with following touch down PCR conditions: *MPLX No. 1* to *3*: 95°C (15min), 10x[94°C (60s), 57°C* (-0.2°C per cycle*; 90s), 72°C (60s)], followed by 13x [94°C (60s), 55°C (90s), 72°C (60s)] and 72°C (30min); MPLX No. 4: 95°C (15min), 10x [94°C (60s), 52°C* (-0.2°C per cycle*; 90s),

72°C (60s)], 13x [94°C (60s), 51°C (90s), 72°C (60s)], 72°C (30min) (see also Tab. 2 for the respective annealing temperatures and primer pairs assigned to the MPLX number (*MPLX No*.)). A long final elongation of 30 min. is of priority to assure adenylation of all PCR products and so, equal lengths for identical alleles in the fragment analysis. MSAT fragments were amplified under the latter mentioned 4 multiplexing conditions for all samples chosen for the analysis of the *Pocillopora* sp. PCR products were diluted 1:25 prior fragment analysis.

Fragment lengths were detected using the ABI-3730xl Analyzer machine and the ABI files were imported to GeneMapper 4.0 to proceed with genotyping. Alleles were manually and independently scored at least four times each. Ambiguous results were re-amplified with higher DNA content and/or lower annealing temperature and alleles of the different PCR setups then compared for accurate and consistent allele scoring. To check for PCR artifacts like e.g. large allele dropout or presence of null alleles due to high annealing temperatures and small primer-template mismatches, at least 96 PCR setups were repeated 2-3 times at lower and higher annealing temperatures. After independent repeated genotyping, the allele scoring of the different PCR setups were compared for each sample.

3.4.3 Data Analysis

Genotypes from GeneMapper 4.0 were imported to Excel were the MICROSATELLITE EXCEL TOOLKIT 3.1.1 (Park 2001) was used to detect missing (diploid) and invalid/incredible data (e.g. alleles, population names, data format and characters). This Excel tool was also used to find clonal colonies within the data set by detecting matching multi-locus-samples allowing up to 4 mismatches. The corrected data set was converted and exported in GENEPOP TEXT file format.

For further statistical analysis, the program CONVERT 1.3.1 (Glaubitz 2004) and PDG SPIDER 2.0 (Java front) (Lischer and Excoffier 2012) were employed to convert the data file into different file formats.

MICROCHECKER 2.2.3 (Shipley 2003) was used to detect presence of null alleles, errors in allele sizes due to stuttering and large allele dropout by comparing observed and expected homozygote and heterozygote frequencies.

Population differentiation on *genic* and *genotypic* level was performed for all

population pairs with GENEPOP 4.1 (Raymond and Rouset 1995). Both tests were run using the Markov Chain (MC) algorithm by Gou and Thompson (1992) with 10,000 burnin, 100 batches and 5,000 MC Monte Carlo (MCMC) steps. Corrected p-values were used to assign the significance of differentiation according to the F_{ST}-values, also calculated with GENEPOP 4.1.

Other applicable measurements of differentiation were calculated with GENALEX 6.5 (Peakall and Smouse 2006, 2012), e.g. differentiation coefficient *D,* G_{ST}*,* G'_{ST}, effective number of alleles N_e*,* pairwise fixation indices F_{ST}, Nei's genetic identity I_N and distance D_N. Assignment tests and visualization of data were also performed with this EXCEL based macro package.

STRUCTURE 2.2.3 was employed (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009) to assess population structure based on Bayesian multi-locus clustering. The number of putative subpopulations (*K*) based on the STRUCTURE analysis was calculated with STRUCTURE HARVESTER's *Evanno Method* (Earl and vonHoldt 2012). Prior runs of STRUCTURE were performed using 100,000 MCMC steps and 50,000 burn-in for *K=1* to *K=10* (maximum number of sampled reefs) in order to firstly infer the range of the possible population numbers. For the final iterations, *K* was set from 1 to 4 with 1,000,000 MCMC and a burn-in period of 500,000. These conditions were again tested for all possible models using the entire sample set (n=252) without giving the sample location as a prior. Results from all models were compiled before using STRUCTURE HARVESTER to give the less bias answer on the total number of detected subpopulations.

Final assessment of the population's state, differentiation, size and migration was performed with the software MIGRATE-N 3.5.1 (Beerli 2006), which estimates parameters using Bayesian inference implementing marginal likelihood estimations in two different manners (instead of using only Makov Chains Monte Carlo MCMC and likelihood ratios): (1) The harmonic mean (Newton and Raftery 1994; Kass and Raftery 1995) and (2) path sampling (Gelman and Meng 1998); latter with and without thermodynamic integration (Gelman and Meng 1998; Lartillot and Philippe 2006; Friel and Pettitt 2005, 2008; for more information see also Beerli and Palczewski 2010). This program allows the user to define multiple parameters for the calculation of the probability of a certain model on population connectivity and migration. In comparison

to former programs, which base the inference of population connectivity on a set model (e.g. stepping stone or island model), MIGRATE-N allows the user to model the putative migration routes and rates discretely to compare different hypotheses on connectivity. Thus, it is currently the most powerful tool to freely and accurately estimate the probability of a variety of plausible migration models between demes/subpopulations with high statistical power.

4. Results

4.1 mtDNA Markers – Preliminary Results

4.1.1 *igr11* - Putative Control Region (*CR*)

From an expected ~1,250 bp long locus of the mtDNA *CR,* between 700-850 bp (using RCOI-3') and 350-480 bp (using FNAD5.2deg) long sequences were yielded. Therefore, the contig of bi-directional sequences could not be build for all samples when there was no overlapping sequence between the two (F+R) sequence's fragments because the consensus sequences were not long enough. The main reason for the relatively short sequence reads, mainly observed for the *FNAD5.2* primer, is probably *A/T* rich regions with long *A* or *T* homopolymers. During sequencing, the high number of *T* and/or *A* repeats right at the beginning of the locus probably induced an exponential decrease of these two types of nucleotides in the BigDye. This reduces the availability of one or both nucleotides as the sequences increase in length. As a consequence, reduced sequence length and signal are induced by the limited availability of *A* and/or *T* nucleotides (ddATPs and/or ddTTPs). Furthermore, and mainly in the case of long sequences with many repeats of a single nucleotide (homopolymers) as present in the *CR*, it is more likely for errors like e.g. polymerase slippage and/or stuttering to occur. This type of errors increase the number of deletions and insertions in the regions of single nucleotide repeats, which increasing the amount of ambiguous peaks downstream in the sequence. Thus, the quality of the sequence decreases.

Former mentioned limiting nucleotide availability during the sequencing reaction was mainly detected in the reverse sequence of *CR* and could be solved by changing the ddNTP ratios of the Big Dye or lowering the concentration of the template PCR product (BCL and Sanger Sequencing Troubleshooting Guides). However, overcoming the latter mentioned disturbing factor is much harder.

As a possible solution, a different primer pair Rs-F15 and Rs-R15 (Lin *et al.* 2011) was used to sequence the same locus from a different starting point at the *CR*, hoping to avoid some of the disturbing A/T homopolymer regions. However, only the reverse primer Rs-R15 yielded a high quality sequence (800-950bp).

Because difficulties during sequencing were encountered (only approx. 800 bp F + 300 bp R/~1,250bp) one could assume that some of the variability of this locus might be lost since not the complete 1,250 bp sequence was available for most samples.

Nevertheless, high quality sequences of the last 800 bp and at least first 200 bp of the *CR* could be over all yielded and thus, one should expected to capture and resolve most or the least some of the variability of this locus. Conversely, no base pair differences could be detected in the alignment between any of the sequenced 83 individuals. Subsequently, this locus was discarded for the genetic distinction of further samples.

4.1.2 *ORF* – Open Reading Frame

The same subset of samples that was used to check for variability in the *CR,* was also used for sequencing of the *ORF*. Generated sequences were about 850 bp long. In the case of the *ORF*, single base pair mutations could be clearly identified in Codon Code Aligner. Therefore, this marker was chosen to identify single mitochondrial lineages and rule out possible bias in further analysis due to inclusion of cryptic species. Consequently, all available 365 samples were sequenced for this locus.

Sequences were independently checked for ambiguous peaks or low quality sequence and the contig of the bi-directionally sequenced locus was built. No ambiguous peaks were detected and no low quality sequences were included in the alignment. DNasp generated haplotype data for 6 distinct haplotypes (A to F), equally present among all sampled reefs. Preliminary analysis of the haplotypic data in MEGA suggested the grouping of haplotypes A-E and a second cluster solely constituted by haplotype F. Samples from the F haplotype were excluded, in order to confidently exclude possible bias due to the inclusion of cryptic species in the genetic analysis of populations.

By means of this preliminary analysis 113 samples with haplotype F were excluded, resulting in a sample set of 252 samples for the connectivity assessment of the population of *Pocillopora* sp. Originally samples taken for this study were referred to as *Pocillopora verrucosa*. However, since morphology seems to be a weak mean of species differentiation in the case of *Pocillopora* (Pinzon *et al.* 2013) and many other corals (West *et al.* 1993; West 1997; Bruno and Edmunds 1997; Muko *et al.* 2000), the species used in the following analysis will be referred to as *Pocillopora* sp. In this regard, it is worth mentioning that there were no evident morphological differences observed between the putative different mitochondrial lineages (A-E and F). For this purpose, photographical vouchers of the specimen sampled were assigned to different

morphotypes before molecular data on the *ORF* was available to assure independent and un-skewed morphological analysis. This assignment of specimen to groups by means of morphological macro features (e.g. Colony structure and polyp arrangement) was independently repeated three times to check for consistency in the method. If e.g. sample *x* was identified as morphotype *a* but later two times as morphotype *b* it was called *a/b/b* in order to record as much information as possible on morphological differentiation between the two mitochondrial lineages. After the haplotypic data was available, results from the macro-morphological and molecular analysis was compared but no correlation between both data types was found. Additionally, vouchers of samples with F haplotype were reexamined separately for congruent features differing from the other samples (with haplotypes A-E). Once again, no morphological feature at the macroscopic level could be found to differentiate both mitochondrial clusters (A-E and F). Moreover, morphotyes were rather scattered between the 2 putative mitochondrial lineages and the distribution of all six mitochondrial lineages was also homogeneous for every sampled reef and site (see Tab. 1).

4.2 Microsatellite nDNA Markers

4.2.1 Primer Selection and Literature Consultation

A total of fourteen MSAT primers were found in former literature (see Tab. 2) and tested on *Pocillopora* sp. from the Red Sea.

Pd10 was discarded, as it did not yield any PCR product under various annealing temperatures. Possibly, the targeted genomic primer region is not conserved between the *Pocillopora* species and thus, hybridization could not occur and the locus did not amplify.

Solely microsatellite markers (MSATs) were targeted, although other marker types have been used for similar studies. For example other markers like internal transcribed spacer ribosomal DNA (ITS or ITS2) seem to have difficulties concerning the type of data they provide and how it should be interpreted and analyzed. The ITS marker often is a multiple copy gene (due to e.g. pseudo-genes) with large intraindividual polymorphisms, which makes the interpretation of an analysis of species or population differentiation based on this marker very difficult (Souter at al. 2010).

Literature was also consulted regarding the HWE and LD for the microsatellite loci implemented in this study. PV7 was mentioned to possibly be a marker under selection, since it is located in the intron of ITS region (Combosch *et al.* 2008, Souter 2010). This was taken into consideration for later analyses and was assessed by inferring some statistics (in STRUCTURE) only based on the locus PV7 as well as after its exclusion on the rest of the data set, to see how results are influenced by this locus' presence in the data. Nonetheless, the marker was incorporated in upcoming analyses since no notable bias could be detected. Additionally, having a large marker set (i.e. more than 6 loci) moderates the influence of a single locus and counteracts deceiving influences of single markers, delivering reliable results.

In this regard, it was remarkable to notice how different authors used different marker sets with usually low number of total microsatellite loci (around 6 loci per study) even though there are more than 14 microsatellite markers published. None of the authors truly gave an explanation for the relative low number of markers employed in the population analysis (Souter *et al.* 2009; Starger *et al.* 2010; Yeoh and Dai 2010; Pinzon and LaJeunesse 2011; Combosch and Vollmer 2011; Pinzon *et al.* 2012; Schmidt-Roach *et al.* 2012). Therefore, the following results concerning each of the tested MSAT loci can be taken as a guide when applying same primers in future studies on *Pocillopora* spp.

4.2.2 Amplification, Fragment Analysis and Genotyping

After successful standardization of PCR conditions for the remaining 13 primers, locus Pd3 had to be excluded. The fragment analysis for this locus showed ambiguous and multiple fragment amplification, which could not be detected on the QIAXCEL machine beforehand.

Fragment analysis of repeated samples under different PCR conditions gave the same results discarding the possibility of allele dropouts as a result of PCR artifacts like e.g. primer combinations in the multiplex reaction and primer annealing temperatures. Independent and repeated manual allele scoring also presented consistent genotypes in all cases. Primers Pd1, Pd4, Pd5, Pd6, Pd7, Pd8, Pd9, PV2, PV5, PV6, PV7 amplified

consistently the same allele lengths for the samples and thus, no missing or ambiguous data was present in the study.

4.2.3 Data Analysis

Erratic values (e.g. typos) in the genotype data of 12 MSATs markers were corrected after using MICROSATELLITE EXCEL TOOLKIT. This program was also employed to detect possible clones within the samples based on identical multi-locus genotypes. No clones were present in the data set.

MICROCHECKER revealed probable presence of null alleles, allele scoring errors due to polymerase stuttering and significant deviations from HWE for Pd2 and PV2. Looking at Tab. 1 the high number of alleles (48) genotyped for PV2 are also an evidence for a suspicious behavior of this locus. Relative wide ranges of fragment lengths can also be interpreted as dubious MSAT analysis' results. Moreover, if the MSAT marker has a very short short tandem repeat (STR) motif (e.g. dinucleotide repeat) and low allelic diversity, like it is the case for Pd2, lower ranges of different fragment lengths should be expected. Pd2 and PV2 were herewith expelled from the data set.

Overall, MICROCHECKER could not find any signs of large allele dropout. For all remaining 10 microsatellite loci, neither biasing artifacts of PCR, nor scoring errors, or deviations from expected values were detected. The remaining data set is referred to as *"10loci"*, consisting of Pd1, Pd4, Pd5, Pd6, Pd7, Pd8, Pd9, PV5, PV6 and PV7.

A preliminary run of STRUCTURE was performed with *"10loci"* without giving sampling population as prior information, in order to infer putative number of populations *K*. STRUCTURE HARVESTER indicated $K = 1$ as the most likely total number of populations for *"10loci"*. Whenever *K* was set higher (≥ 2), individuals were homogeneously assigned to *K* number of clusters (= putative populations), with more or less same probabilities of corresponding to any of the *K* number of clusters (see Fig. 3). Latter observation is also an indicator for the lack of genetic structure among the sampled demes. This preliminary test suggests panmixia among sampling sites. Such

preliminary information on the population's genetic structure is of importance in order to perform the upcoming statistical tests and was used to avoid erratic analysis setup due to misleadingly applied statistics and for example, the Wahlund-Effect.

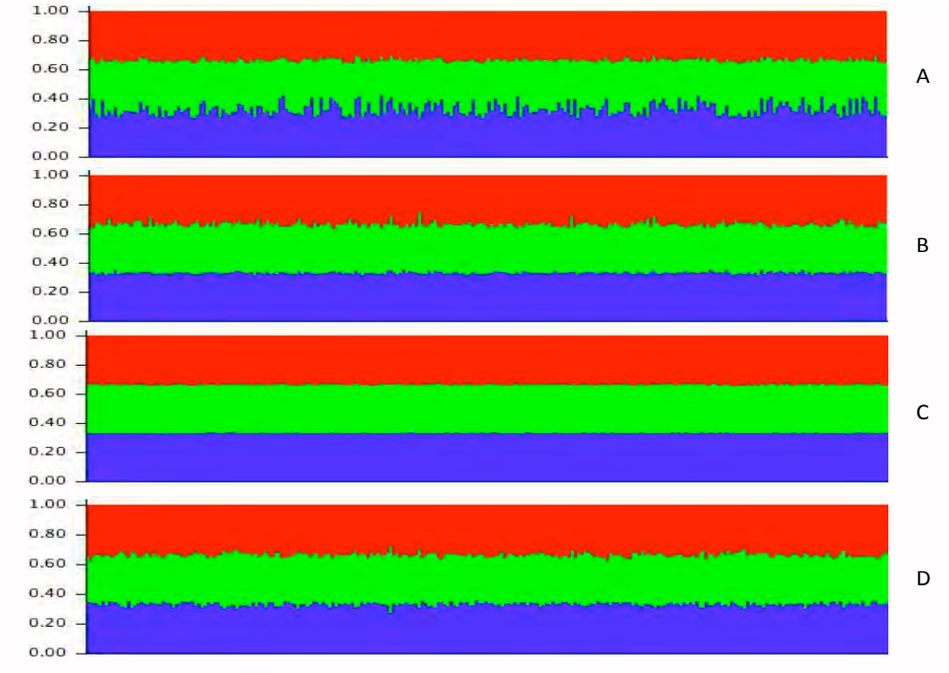

Fig. 3: STRUCTURE clustering: Samples are on the *X-axis* (each individual represented by a single vertical line; The ancestry vector Q is displayed on the *Y-axis*; the colors represent the number of possible clusters (in this case three because *K*=3, with *K*= number of putative populations); The proportion of estimated membership for each individual, in each cluster is represented by the color partitioning of each individual vertical line. Models applied for the population assignment are coded on the right-hand side with the letters A-D. A and B: "no admixture model" (samples belong discretely to either one or another population from *K*); C and D: "admixture model" (each sample has partially genomic information each of the K populations ≠ complete isolated populations). A and C: models are implemented with "correlated allele frequencies" (frequencies are rather similar between different populations); B and D: models with "independent allele frequencies" (allele frequencies in different populations are considerably different).

Subsequently, basic statistics were implemented in GENEPOP over all samples (*K* =1) based on *"10loci"*. No significant linkage disequilibrium (*LD*) among loci pairs (MC parameters: 10000 dememorization, 1000 batches and 10000 iteration steps per batch) was found except between Pd1 and Pd8 (0.0021±0.0003 with log likelihood ratio statistic and 0.0019±0.0003 for probability test). Since previous studies on *Pocillopora* spp. never reported significant *LD* between these two loci (Starger *et al.* 2008; Souter *et al.* 2009; Starger *et al.* 2010; Combosch and Vollmer 2011) and since this *LD* was not found if looking at single sampling sites (WAJ, KAU and DOG), these loci were kept

for further analysis.

Illustrations of allele distributions per locus implemented with GENALEX 6.5 (Peakall and Smouse 2006, 2012), showed normally distributed allele lengths frequency for nine out of the ten remaining loci. This was relatively consistent also for sample sets divided in 3 or 10 populations according to the sampling location (*3pops*; *10pops*). Locus Pd5, however, showed allele length frequencies distributed around three mean allele lengths (Fig. 4). This indicates that either Pd5 primers are potentially amplifying at least two different loci (which is crucial for its reliability and the analysis) or that this locus shows a multi modal allele frequency due to e.g. variability in its flanking regions. That would lead to the "jump" from one virtual length to another for basically the same allele identity. In both cases the homology of this locus is doubtful and has to be queried. It is important to stress in this context, how simple visualization of data can help reveal other issues blind to statistical approaches. To make sure that altering data is not corrupting the analysis, Pd5 was completely excluded from the data set for the following analysis.

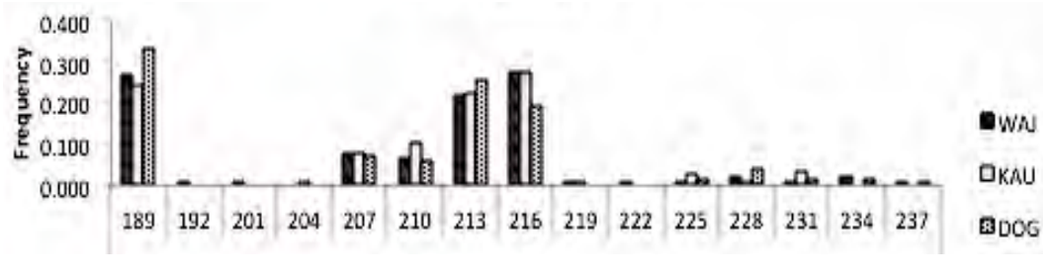

Fig. 4: Allele length frequencies of *Pd5* for the three sampling sites of WAJ, KAU and DOG; generated by GENALEX 6.5 (Peakall and Smouse 2006, 2012).

GENEPOP was also used to calculate exact p-values for tests on heterozygote deficit (= alternative hypothesis, H1; with former MC parameters), which were significant for Pd7 after Bonferroni correction (p: 0.0000±0.0000; F_{IS}-values: 0.0463 (Weir and Cockerham 1984, "W&H") and 0.13 (Robertson and Hill 1984, "R&H"). Based on this result and the wide range in allele sizes for this locus (162-352 bp length), as well as literature reporting deviation from HWE (Starger *et al.* 2008), Pd7 was provisionally excluded to offer a conservative data set.

Consequently, the following analyses and tests are performed with and without the inclusion of Pd7. Results based on the dataset including Pd7 are of interest since

they include much information on genetic differentiation as Pd7 seems to be a highly variable marker (15 alleles, see Tab. 2) within this study and hence, has potentially higher resolution on more recent (ecological timescale) population structure. Besides, many other studies have used this marker without reporting issues (Yeoh and Dai 2010; Pinzon and Lajeunesse 2011; Pinzon *et al.* 2012; Schmidt-Roach *et al.* 2012). Whenever the dataset including this marker is used, it will be referred to as "*9loci*" (i.e. Pd1, Pd4, Pd6, Pd7, Pd8, Pd9, PV5, PV6 and PV7). The second data set excluding Pd7, which will also be used in all following analyses, will be referred to as "*8loci*". Latter will serve as a control to see how Pd7 might be influencing the analysis and to compare results to detect and report bias induced by the Pd7 MSAT.

STRUCTURE was once again consulted as a prior tool to assess the putative number of populations among the samples after exclusion of Pd5 ("*9loci*") and also of Pd7 ("*8loci*"). STRUCTURE HARVESTER (Pritchard *et al.* 2000) indicated once more $K = 1$ to be the most likely total number of populations (see Tab. 3). However, one should keep in mind that whereas it is easy to detect the most appropriate number of K when dealing with more than 2 putative populations, the differentiation between panmixia ($K = 1$) and two populations ($K = 2$) is the most difficult to make. One of the main reasons is that there is no comparative *LnP(K)* value for $K = 0$ to deduce Delta K (Evanno method; Tab. 3).

Tab 3: STRUCTURE HARVESTER - Putative Population Numbers (Evanno Method)

	K (No.)	Repeats	Mean *LnP(K)*	SD *LnP(K)*	*Ln'(K)*	*|Ln''(K)|*	*Delta K*
8loci	***1***	20	***-4641.115***	0.719	-	-	-
	2	20	-4734.130	113.808	-93.015	38.465	0.338
	3	20	-4865.610	192.568	-131.480	16.595	0.086
	4	20	-4980.495	281.443	-114.885	-	-
9loci	***1***	20	***-5322.830***	1.075	-	-	-
	2	20	-5392.300	83.581	-69.470	67.730	0.810
	3	20	-5529.500	172.489	-137.200	24.780	0.144
	4	20	-5641.920	214.402	-112.420	-	-

Results of the Bayesian clustering analysis by STRUCTURE are based on 8 ("***8loci***") and 9 loci ("***9loci***") (first column). The inference of total population numbers by STRUCTURE HARVESTER is based on 20 STRUCTURE runs (=**Repeats**). Calculated values are: the mean logarithm of the probability of K possible populations (**Mean *LnP(K)***), its standard deviation (**SD *LnP(K)***), first and second derivative (***Ln'(K)*** and ***Ln"(K)*** respectively) and difference between probabilities for two possible total *K*'s (***Delta K,*** *Evanno method*). ***Bold*** values represent the most likely total *K* of the data set.

GENEPOP's inference of *F* indices and *Rho* coefficients, as well as genetic differentiation for 3 (geographic sites) and 10 (reefs) total populations was repeated for "*8loci*" and results were compared to those from "*9loci*" (Tab. 4).

Inference of F indices based on ANOVA by GENEPOP (Tab. 4), overall, shows that F_{IS} values are lower than F_{IT}; and F_{ST} values are the lowest of all three. Respectively, Rho correlation coefficient (based on allele size) is higher between individuals from a subpopulation (Rho_{IS}) than between all individuals (Rho_{IT}) and the correlation between subpopulations (Rho_{ST}) is negative and/or very close to 0; i.e. not existent. This means that allele sizes of subpopulations have no explanatory value regarding the variance of the total data. In other words, grouping individuals according to their sampling sites, either per reef (*10pops*) or per geographic site (*3pops*) does not decrease the variance of the data, indicating that there is no correlation between the genetic variance and the geographic location of the sample, and therefore no genetic differentiation between the sampling sites (neither for reefs nor geographic sampling regions).

Tab. 4: **ANOVA Results for the inference of F indices by GENEPOP after Weir and Cockerham (1984)**

	F_{IS}	F_{ST}	F_{IT}	Rho_{IS}	Rho_{ST}	Rho_{IT}
3 pops:						
10loci	0.0152	0.0006	0.0158	0.1544	-0.0057	0.1495
9loci	0.0193 (*0.087*)	0.0005 (*0.340*)	0.0198 (*0.079*)	0.1995* (*0.001*)	-0.0069	0.1940* (*0.001*)
8loci	0.0153 (*0.177*)	0.0009 (*0.258*)	0.0162 (*0.161*)	0.0823 (*0.005*)	-0.0025	0.0800 (*0.006*)
Variance	***Among Inidv***	***Among Pops***	***Within Indiv***	***Among Inidv***	***Among Pops***	***Within Indiv***
9loci	1.9%	0.0%	98.0%	8.2%	0.0%	91.8%
8loci	1.5%	0.1%	98.4%	8.2%	0.0%	91.8%
10 pops:						
10loci	0.0155	0.0001	0.0156	0.1545	-0.0044	0.1508
9loci	0.0195 (*0.098*)	0.0001 (*0.461*)	0.0196 (*0.094*)	0.2004* (*0.001*)	-0.0064	0.1953* (*0.001*)
8loci	0.0149 (*0.157*)	0.0012 (*0.332*)	0.016 (*0.145*)	0.0777 (*0.010*)	0.0038 (*0.240*)	0.0812 (*0.009*)
Variance	***Among Inidv***	***Among Pops***	***Within Indiv***	***Among Inidv***	***Among Pops***	***Within Indiv***
9loci	2.0%	0.0%	98.0%	20.0%	0.0%	80.0%
8loci	1.5%	0.1%	98.4%	7.7%	0.4%	91.9%

First column: different data sets for the calculation of F- and R-statistics of the ANOVA (Analysis of Variance). **3 pops:** The ANOVA is based on data divided in 3 populations (pops) according to the geographic location (i.e. WAJ, KAU, DOG); **10 pops:** The ANOVA is based on data divided in 10 populations according to the reef of origin (4xWAJ, 3xKAU, 3xDOG). ***10loci, 9loci*** and ***8loci***: Number of loci included in the ANOVA. **Variance**: percentage of total variance within the Data explained by the statistical analysis/approach. **F_{IS}:** estimator of differentiation between *Idividuals* (**I**) and *Subpopulations* (**S**), **F_{ST}:** between subpopulations and the *Total* population/samples (**T**), **F_{IT}:** among all individuals (between individuals of the total data set). **Rho** values are named accordingly.

Genic and *genotypic* differentiation between the populations for 3 and 10 putative populations was calculated with GENEPOP. Genic differentiation for *3pops* is only significant, if calculated per locus, between KAU and DOG samples for Pd9 (*0.0048*±0.0006 S.E.) and PV5 (*0.0173*±0.0021 S.E.) based on "*9loci*" as well as based on *"8loci"* (Pd9 (*0.0066*±0.0009 S.E.); PV5 (*0.0204*±0.0025 S.E.)). However, there is no significant genic differentiation between populations based on multi-locus analysis neither for "*9loci*" nor *"8loci"*.

Similarly, significant genotypic differentiation for single loci was computed for "*9loci*" (for Pd9 (*0.0105*±0.0014 S.E.) and PV5 (*0.0286*±0.0032 S.E.)) and *"8loci"* (for Pd9 (*0.00924*±0.00131 S.E.); and PV5 (*0.0277*±0.0024 S.E.) between KAU and DOG, but there was no significant genic differentiation between sampling sites when calculated based on multi-locus data.

However, when discriminating between reefs (*10pops*), significant genic differentiation over all loci (i.e. multi-locus estimations) was found between KAU1 and DOG1 (*0.0013*, "*9loci*"; *0.0009*, *"8loci"*) and between KAU3 and DOG1 (*0.0003*, "*9loci*"; *0.00003*, *"8loci"*) even after Bonferroni correction (in *italic*). Significant genotypic differentiation was also present between aforementioned populations, with only differentiation between latter population pair being significant after Bonferroni correction (i.e. KAU1/DOG1 (0.0060, *"9loci"*; 0.0047, *"8loci"*); KAU3/DOG1 (*0.0011*, *"9loci"*; *0.0007*, *"8loci"*).

Number of migrants (*Nm;* GENEPOP) between reefs (*10 pops*) based on *"8loci"* (14.5943, corrected for population size) was higher than based on *"9loci"* (9.0800, corrected for population size), which implies increased genetic structure and differentiation of the populations (established by less migration between populations) when taking Pd7 into the analysis. The same observation was made when assuming three demes (*3 pops*; WAJ, DOG, KAU) with *Nm* of 20.2126 ("*9loci*") and 29.8392 ("*8loci*"). This supports the postulation of higher resolution of differentiation at the ecological timescale when accounting the putative faster evolving Pd7 marker (due to high mutation rates). Raw estimates of numbers of migrants are displayed in Tab. 5 and are based on the mean Shannon Mutual Information Index (sH_{UA}) calculated by GENALEX 6.5. Furthermore, migration seems much higher between population pairs WAJ and KAU (139.669 (9loci)/ 129.87 (8loci)) compared to number of migrants for

population pairs with DOG (DOG and WAJ: 83.272 (9loci)/65.906 (8loci); DOG and KAU: 65.545 (9loci)/58.393 (8loci); see Tab. 5). These estimates of migrations might give a hint on partially limited gene flow from and to DOG.

Tab. 5: GENALEX 6.5 - Shannon Diversity Indices and Migration Estimates

	sH_{UA} \ sH_U; \=sH_A			N_m	
9loci	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**
WAJ	1.165	1.155	1.172	-	-
KAU	0.009	1.127	1.150	139.699	-
DOG	0.013	0.014	1.150	83.272	65.545
8loci					
WAJ	1.150	1.139	1.148	-	-
KAU	0.009	1.111	1.127	129.872	-
DOG	0.012	0.013	1.116	65.906	58.393

Estimation of Shannon indices and migrants ($\mathbf{N_m}$) (Shannon 1948) is based on either 9 (**9loci**) or 8 loci (**8loci**). $\mathbf{sH_{UA}}$**:** Shannon's Mutual Information Index (bellow the diagonal); $\mathbf{sH_U}$**:** (above the diagonal); sH_A: Shannon's allelic diversity index (along the diagonal, "**\=**")

Additionally, tests on population assignment based on "*8loci*" show proportionally higher assignment of individuals from DOG to their population of origin itself (DOG) than to another relatively to the assignment test results for KAU and WAJ (Tab. 6). DOG might be relatively genetically more isolated from the total *Pocillopora* sp. population in comparison to KAU and WAJ. However, the isolation is not significant in order to reject panmixia. In general, WAJ showed the highest proportions of assignment to other populations, indicating putative high gene flow to and from this population (Tab. 6). Increased gene flow from WAJ can also be deduced from the preliminary MIGRATE-N model B (see following results and Fig. 3).

Tab. 6: GENALEX 6.5 - Population Assignment Test

	Self	Other	Proportion
WAJ	20	68	0.29
KAU	36	63	0.57
DOG	26	39	0.67
Total	82	170	0.48
%	33	67	

Samples from 3 geographic locations (WAJ, KAU and DOG) are assigned to either its own or another of the 2 putative populations

Estimation of other differentiation parameters, which seem more adequate than F-statistics when using microsatellite based data for population's connectivity analysis, were performed in GENALEX. Even though, F_{ST} values calculated with AMOVAs are treated as a hypothesis about differentiation within the population and among its constituting demes, the interpretation of such as an indicator of differentiation has lead to various discussions mainly if applied on data from highly variable loci (e.g. Hedrick 1999, 2005; Balloux *et al.* 2000, Jost 2008, Meirmans and Hedrick 2011). In general it is helpful to continue to work with F-indices, since most studies have based their results on these allowing a comparison with previous studies. However, e.g. Jost (2008) and many others (Hedrick 2005; Meirmans and Hedrick, 2011; Blyton and Flanagan 2012 – GENALEX 6.5 Guide by Blyton and Flanagan 2006-2012) have proposed the utilization of more adequate differentiation indicators like D_{EST}, G'_{ST} and unbiased Nei's distance (*uD*) and identity (*uI*) coefficients. Latter two give particularly good results for population analyses based on microsatellite data (Takezaki and Nei 1996). Nevertheless, in the case of our study none of those estimators were able to capture or resolve differentiation of the demes (*3pops* and *10pops*). Hence, same conclusions about wide gene flow between sampling sites made from the ANOVA's F_{ST}-values also hold true for the more current used parameters (Tab. 7).

Tab. 7: Coefficients for Genetic Differentiation Assessment between Populations Pairs

	D_{EST} \ *p*			F_{ST} \ *p*			R_{ST} \ *p*			G'_{ST} *N* \ *p*			G'_{ST} *H* \ *p*			*uI N* \ *uD N*		
9loci	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**	**DOG**	**WAJ**	**KAU**	**DOG**
WAJ	\	0.865	0.206	\	0.454	0.207	\	0.310	0.322	\	0.865	0.206	\	0.002	0.002	\	0.000	0.002
KAU	-0.002	\	0.132	0.000	\	0.109	0.000	\	0.353	-0.002	\	0.132	*-0.003*	\	0.002	1.002	\	0.003
DOG	0.002	0.003	\	0.002	0.002	\	0.000	0.000	\	0.002	0.002	\	*0.003*	*0.004*	\	0.998	0.997	\
8loci																		
WAJ	\	0.797	0.142	\	0.799	0.142	\	0.396	0.381	\	0.797	0.142	\	0.002	0.002	\	0.000	0.003
KAU	-0.002	\	0.102	0.002	\	0.100	0.000	\	0.387	-0.002	\	0.103	*-0.003*	\	0.002	1.002	\	0.004
DOG	0.003	0.004	\	0.005	0.005	\	0.000	0.000	\	0.002	0.003	\	*0.004*	*0.005*	\	0.997	0.996	\

Estimation of differentiation is based on either 9 (**9loci**) or 8 loci (**8loci**). **G'_{ST} N** (Nei, 1983), **G'_{ST} H** (Hedrick, 2005) **and D_{EST}** (Jost, 2008) are differentiation indices (bellow the diagonal); ***p***: is the respective probability value (above the diagonal). **uI N:** Nei's unbiased genetic identity measure (bellow the diagonal); **uD N:** Nei's unbiased genetic distance measure (above the diagonal) (Nei 1972).

To evaluate if panmixia is the most appropriate model explaining the genetic information of *Pocillopora* sp. in the Red Sea, MIGRATE-N was run for different parameter settings. The purpose of this last analysis is grounded on the probability of a wrong assumption of panmixia due to models embedded in calculations performed by previous employed tools of genetic differentiation and structure. Pre-established models are for example, (a) "stepping stone model" (Kimura 1953), which assumes that in each generation an individual can migrate at most one step in either direction between demes (i.e. exchange of individuals is restricted between adjacent colonies); or (b) "island model" (Moran 1959). In other words, since the conventional programs make insinuations of the population structure based on set parameters for specific models, if those prearranged models do not explain the data adequately, panmixia would result as the most suitable model compared to any of the prior mentioned. However, if one is able to decide within the application, which model to test, one can compare the likelihood of the different assumed models and make a supposition of the best fitting migration route. However, the more parameters you are able to manipulate, the more time consuming it is to adjust sensible ranges for each parameter (e.g. different population sizes for each population, asymmetric migration rates and discrete sink and source populations). Additionally, MIGRATE-N uses Bayesian inference as well as maximum likelihood increasing the demand of random access memory and the time required for single runs per model. Thus, the initial establishment of the suitable parameter settings is increasingly time consuming. Therefore, the following results based on MIGRATE-N are preliminary and might change after further adjustment of the

input parameter ranges.

Tab. 8: Model of Migration Routes implemented for MIGRATE-N

A)	WAJ	KAU	DOG
WAJ	*	*	*
KAU	*	*	*
DOG	*	*	*

B)	WAJ	KAU	DOG
WAJ	*	0	0
KAU	*	*	0
DOG	*	*	*

C)	WAJ	KAU	DOG
WAJ	*	*	0
KAU	*	*	*
DOG	*	*	*

D)	WAJ	KAU	DOG
WAJ	*	*	0
KAU	*	*	*
DOG	0	*	*

E)	WAJ	KAU	DOG
WAJ	*	0	0
KAU	*	*	*
DOG	0	0	*

BB)	WAJ	KAU	DOG
WAJ	*	*	*
KAU	0	*	*
DOG	0	0	*

CC)	WAJ	KAU	DOG
WAJ	*	*	*
KAU	*	*	*
DOG	0	*	*

FF)	WAJ	KAU	DOG
WAJ	*	*	*
KAU	*	*	*
DOG	0	0	*

Three geographic sites are displayed as a matrix (WAJ, KAU, DOG); Migration between two sites is symbolized by a star (*). A star (*) always implies migration in one direction, from the population at the top of the matrix (X-axis) to the population to the left hand of the matrix (Y-axis). Each different migration model is named with a capital letter. Matrices named with two capital letters have not been yet tested.

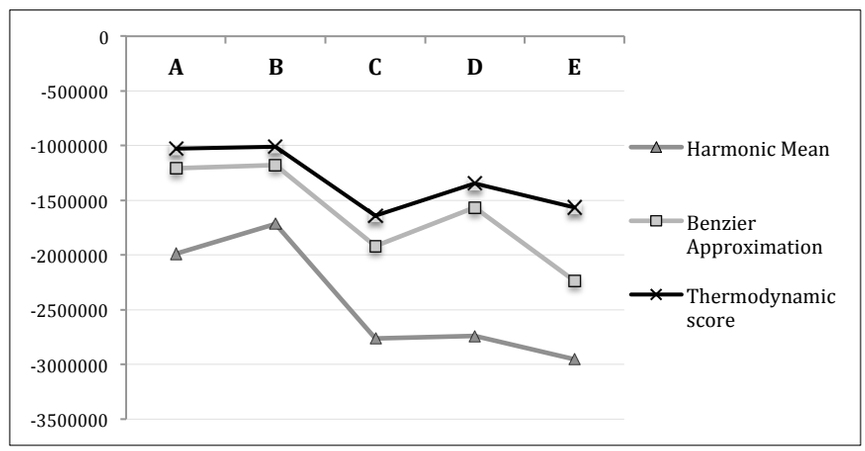

Fig. 3: MIGRATE-N: Output of the marginal likelihood inference of models A to B from Tab. 8. All three displayed values indicate that model A and B explain the input data the best.

5. Discussion

5.1 mtDNA – Preliminary Analysis

5.1.1 mtDNA Marker – Background Information

Mitochondrial DNA is widely used for phylogenetic studies among many species (Avise *et al.* 1987; Shearer *et al.* 2008) primarily, due to generally high mutation rates and polymorphism compared to single-copy nuclear DNA (scnDNA) (Brown *et al.* 1979, 1982). However, the evolution of this molecule is not consistent among taxa. Mammals have higher rates of nucleotide substitutions than fish and many invertebrates (Lynch and Jarrell 1993, see also Shearer *et al.* 2008). In fact, some invertebrates have mtDNA mutation rates of similar ranges to those in scnDNA (e.g. Vawter and Brown 1986; Sharp and Li 1989; Lynch and Jarrell 1993; DeGiorgi *et al.* 1996; Metz *et al.* 1998). For example, anthozoan mitochondrial genes have an extreme slow evolutionary pace of about 10-20 times lower than of vertebrates, which have approx. 1-2% sequence divergence per Myr (Brown *et al.* 1979; Ferris *et al.* 1983; Higuchi *et al.* 1984; Wilson *et al.* 1985). In the case of corals for example, the divergence of mtDNA is very small even between different species, which share high number of conserved sequences (Medina *et al.* 1999; Snell 2000; Shearer *et al.* 2008). Due to latter mentioned, it is basically impossible to make intraspecific differentiation based on mitochondrial markers in coral species. Some of the reasons for low evolutionary rate of mitochondria in anthozoan corals are discussed in Shearer *et al.* (2002) and Ridgway and Gates (2006). The most critical feature could be the presence of a homologue component of the bacterial MutSLH mismatch repair pathway found in mtDNA of octocorals (Pont-Kingdon *et al.* 1995, 1998; Beaton *et al.* 1998; France and Hoover 2001, 2002). In yeast, proteins for nDNA repair are transported to mitochondria and seem to have a similar function since mtDNA in this organism also mutates slower than expected in comparison to its nDNA genes (Chi and Kolodner 1994a, b).

Nevertheless, some non-coding mtDNA regions are variable enough and suitable for interspecific differentiation of coral species. As such loci are scarce in corals mtDNA, for the genus *Pocillopora* the complete mitochondrial genome was sequenced to find only two variable regions - the putative D-loop control region (*CR* - igr11) and the *ORF* (Flot and Tillier, 2007).

For the reliability of phylogenetic studies it is recommendable to use as many markers as possible and compare the results for each single genetic marker before

making any assumption. In this regard the ITS was formerly also a marker of choice for phylogenetic analyses in corals. Nonetheless, ITS is now known to have a series of biasing artifacts like e.g. multiple copies in a single individual and overlapping variation not only intra but even interspecific (Vollmer and Palumbi 2004, Combosch *et al.* 2008). Thus, the presently best choice for the assessment of species delimitation is mtDNA markers.

For the preliminary examination of the species haplotypic structure of *Pocillopora* sp., both the *CR* (only patially) and the *ORF* were employed to assure the inclusion of only one single species in the current study.

5.1.2 Study Case and Cryptic Species

A genetic discrimination of morphospecies should always be considered when studying Pocilloporidae. For example Pinzon and LaJeunesse (2011) have found only three genetic lineages as they analyzed seven morphologically distinct species. Similar studies based on the *ORF* haplotypes revealed that the same haplotypes assigned to *Pocillopora* damicornis-like, resulted to be *P*. verrucosa-like in other regions (reviewed in Pinzon *et al.* 2013). The mixture of reproductively isolated lineages among *Pocillopora* spp. is critical for population analyses and might be the reason for many ambiguous results in reproductive mode, clonal structure and incorrect inference of connectivity among subpopulations and hybridization zones among species from former investigations (Flot *et al.* 2010, Souter 2010, Pinzon and LaJeunesse 2011, Schmidt-Roach *et al.* 2012, Pinzon *et al.* 2013).

Having learned from previous studies with erroneous sample sets caused by inaccurate taxonomic resolution, mtDNA markers were consulted for the clear distinction of evolutionary significant units (ESU) among *Pocillopora* sp. Additionally and to avoid further incongruence between morphospecies and genetically defined lineages, the species collected for this study is referred to as *Pocillopora* sp. instead of "verrucosa-like".

5.1.3 Methodological and Technical issues on the *CR*

At first sight the *CR* was expected to resolve most differentiation between the colonies sampled, since it has the highest percentage (*1.5%*) of variability (i.e., highest mutation rates) according to Flot and Tillier (2007). Nevertheless, this locus presents some problems during sequencing. Its high A and T nucleotide content makes it very difficult for the polymerase to perform at its best causing the termination of the sequencing reaction at shorter sequence lengths. A or T repeats are present as long homopolymers in this locus, leading to additional issues like polymerase slippage (Sanger Trouble Shooting Guide, BCL Sequencing Trouble Shooting). It might also be likely for the *CR* as such, to have a secondary structure affecting the sequencing reaction as well, and explaining the presence of homopolymers in the first place. To try to overcome these technical difficulties, R. J. Toonen (Hawai'i Institute of Marine Biology/Coconut Island) was contacted. He has recently worked with this locus to differentiate between Hawaiian *Pocillopora* lineages. Kindly, he forwarded the manuscript of one of his most recent studies employing both, the *CR* and *ORF* loci (Marti-Puig *et al.* in *Prep*.). In the manuscript, from 1,266 sequenced bp for the *CR* only 758 bp were used for the analysis, explained by shorter available sequence lengths from a previous study on this locus. Additionally, even though 59 colonies were used for the analysis, only 37 samples were successfully sequenced for the *CR*, whereas 56 sequences were obtained and analyzed for the *ORF*. This aroused the question, why so little *CR* sequences were analyzed and leads to the assumption that his working-group was facing similar problems when sequencing the mtDNA *CR*. Moreover, after analyzing the *CR* sequences from the Red Sea, strikingly, not a single polymorphic base-pair among all sequences was detected, even though these weren't shorter than the sequences from R.J. Toonen's study. I find it necessary to report the apparent lack of differentiation of the *CR*'s sequences in the Red Sea for the verrucosa-like *Pocillopora* and suggest a revision of the locus variability for specimen worldwide. Furthermore, the design of new primers closer to the putative variable *CR* region would assist future sequencing attempts, by excluding excessive A/T rich sequences and shortening the total sequence for a more accurate analysis.

Likewise, current publications, which used the *CR* to define the species structure within the *Pocillopora* genus, commonly described most differentiation features in the

CR as insertions or deletions of 6-8bp (Souter 2010; Schmidt-Roach *et al.* 2012). Such an observation could not be made within this study either.

5.1.4 mtDNA Lineages Based on the *ORF*

In contrast to the CR, sequences from the mitochondrial *ORF* were easily yielded and had no practical difficulties. Thus, the preliminary phylogenetic analysis was based on this locus and decided which samples were included in the population's genetic analysis of *Pocillopora* sp.

The *ORF* haplotypes were initially blasted against the databank to deduce which species was sampled and if it was concordant with the morphotype verrucosa-like in the Red Sea. Nonetheless, since there is only a single *ORF* haplotype available for *P. verrucosa* and since the available *P. damicornis* haplotypes cannot be reliably categorize as such due to high plasticity within the genus, the species taxonomic name was not specified based on the *ORF* either.

5.1.5 Supplementary Information on Morphology and Conclusions

Photographical vouchers for most of the specimen sampled were clustered into morphotypes based on macro-scale morphology. Within this study as well as in other studies, no correlation could be detected between the putative morphotypes and the *ORF* haplotypes, which is a further evidence to extreme phenotypic plasticity of the genus (Miller 1994; Shaish *et al.* 2007; Todd 2008).

The phylogenetic analysis mentioned under this section (5.) is of supplementary character and was used for the exclusion of putative cryptic species. Thus, it still needs to be extended in order to give information on the number of *Pocillopora* species in the Red Sea. Defined mitochondrial linages and a haplotype tree based on the *ORF* will hopefully be presented within upcoming studies. For this reasons these analyses are not presented in detail in the *Results* section (4.). The Red Sea, its geography and climatic features make it perfectly suitable as an incubator for speciation events and hot spot for endemic biodiversity leaving significant leeway for plenty new taxonomical discoveries

(Weikert 1987; Roberts *et al.* 1992; Baars *et al.* 1995; Getahun 1998; Ngugi *et al.* 2012). Wherefore, we are currently conducting parallel studies on the morphologically and genetically conflicting genus *Pocillopora* in the Red Sea.

5.2 Microsatellite nDNA Markers – Population Genetics

5.2.1 Summary of Results

In this study, the population's structure of *Pocillopora* sp. in the Red Sea was expected to reflect environmental conditions and be driven by temperature and/or salinity changes along the Red Sea basin. Instead, the specimens from Saudi Arabia's coast seem to belong to a single, panmixic population (F_{ST}= 0.0005, *p= 0.340;* Variance among sites= 0.0%, based on "*9loci*"). The largest distance between sampling sites from North (WAJ) and South (DOG) covered over 850 km. However, even between these two sites there is no genetic differentiation, denoting enough gene flow over long distances. Similar results have been observed in East Africa, in which *P. verrucosa* (with broadcast spawning as reproductive mode in this area) also appears to effectively counteract genetic drift over up to 697 km (Souter *et al.* 2009). First studies on the connectivity of *Pocillopora* spp. also came to similar conclusions (Benzie *et al.* 1995; Ayre *et al.* 1997; Ridgway *et al.* 2001; Miller and Ayre 2008a, b) but the markers employed therein (e.g. allozymes) have lower evolutionary resolution in comparison to the fast evolving microsatellite markers. Ayre *et al.* (1997) described gene flow even between sites up to 1,200 km apart, based on data from allozymes for the analysis. Microsatellites moreover, not only provide additional information due to the diploid nature of nuclear markers but also supplement the results by having a high resolution at intraspecific level. In this manner they are able to detect even minor signals of recent population structure (Leese *et al.* 2008; Held 2000; Held and Leese 2007; Held and Wägele 2005; Wilson *et al.* 2007).

5.2.2 Reproductive mode

Discussion over distinct reproductive modes within *Pocillopora* spp. has been an ongoing topic of many studies (Stoddart 1984a, b, 1986; Benzie *et al.* 1995; Ayre *et al.*

1997; Kruger and Schleyer 1998; Adjeroud and Tsuchiya 1999; Miller and Ayre 2004; Whitaker 2006). Basically, there are reports over possible sexual and asexual reproduction in *P. verrucosa* and *P. damicornis* (the two most studied species and common morphospecies in the Red Sea), with either one being predominant depending on the geographic location of the specimen (see Ellis and Solander 1786; Stoddart 1983; Richmond and Jokiel 1984; Stoddart and black 1985; Ward 1992; Ayre and Miller 2004; Sherman *et al.* 2006). *P. verrucosa* is also described to reproduce either via brooding (Stimson 1978) or broadcast spawning (Shlesinger and Loya 1985) depending on its geographical location. Likewise, *P. damicornis* is told to reproduce sexually in both manners (brooding and spawning) even in the same area e.g. western Australia (Ward 1992); but only as a broadcast spawners in the eastern Pacific (Glynn *et al.* 1991). However, these disparities in reproductive mode might be predisposed by the high morphological plasticity within Pocilloporidae. An easier explanation might thus, rather be that single species have a specific reproductive mode, but the mixture of such species leads to reports on mixed reproductive means among one morphospecies (like in e.g. Harriott 1983; Richmond and Jokiel 1984; Stoddart and Black 1985; Wards 1992; Tanner 1996; Miller and Ayre 2004; Yeoh and Dai 2010).

Fortunately, it is a fact to us that *Pocillopora* sp. from the current study reproduces sexually via broadcast spawning thanks to studies and observations form directly the same reefs, as those sampled herein (Bouwmeester *et al.* 2011, and also Fadlallah 1983). Asexual reproduction among these species, however, cannot be ruled out completely. Such can take place by e.g. simple fragmentation (Highsmith 1982) independently of the species reproductive strategy.

Other studies stated that asexual reproduction is a normal effect within marginal populations to maintain their population size (Soule 1973; Herlihy and Eckert 2002; Combosch and Vollmer 11). One assumes that the environmental conditions of populations at the periphery of the species distribution ranges are commonly not optimal and unstable (Soule 1973). Additionally, selective pressure on less adapted recruits coming from the center of the species distribution (i.e. the more beneficially located source populations), disadvantages their settling at the periphery, which leads to increased self-seeding of the peripheral population as well as an increased success of asexually produced recruits (Herlihy and Eckert 2002). These are further factors, which could be shaping coral populations in the Red Sea, too.

Since we assume prevailing sexual reproduction among the present study case, samples were taken from offshore reefs because these are less likely to be geographically marginal habitats. In this manner, we can assure that if we find any genetic structure within the population, it will predominantly be caused by the assessed latitudinal T/S-gradient rather than by the phenomenon mentioned above by Soule (1973). Near-shore reefs were not sampled either because these might be exposed to selective pressure and barriers for gene flow caused by human impact rather than by abiotic temperature and salinity fluxes. Additionally, a longitudinal shore-to-open-ocean physicochemical gradient might as well be present (Benzie *et al.* 1995), which was most likely avoided by consistent offshore sampling.

As we can conclude from the results, this technique of sampling seems adequate for the experimental design since, similar number of effective alleles were inferred among all collection sites (mean 2.735±0.235 ("*9loci*")/ 2.747±0.264 ("*8loci*"), based on F-statistics in GENALEX). The number of effective alleles is an indicator of the population's genetic diversity and denotes that the populations from all three sampling sites (WAJ, KAU and DOG) are similarly genetically divers and therefore, similarly stable. Hence, stressors shaping the population and preconditions for larval settlement are apparently more or less equal among all three regions. This explains furthermore, the high connectivity between sites/reefs, general low differentiation/isolation (Tab. 7 and Tab. 6) as well as, the clonal structure discussed under the upcoming section 5.2.3.

The results form this study also show that larvae are being transported over more than 850 km away from their origin and that these, moreover, successfully settle in the new home reef. But how do larvae and/or gametes make it all the way to another reef?

Studies on oocytes of *P. damicornis* (Kruger and Schleyer 1998; Chávez-Romo and Reyes-Bonilla 2007) have revealed that these are infested with zooxanthellae right before they are released into the water column. Zooxanthellae are the corals' symbionts and their main source of energy. Thus, the gametes are perfectly suited for long distance journeys (Tomascik and Sander 1987) similar to the ones assessed for *Pocillopora* sp. Richmond (1987) for example, reports survival of coral larvae for over 100 days before settlement. The distances these larvae can be drifted during such a period of time adds up to the distance already overcome immediately after spawning by the simple drift of the gametes (before the development of the larvae itself).

5.2.3 Clonal Structure

Asexual reproduction among *Pocillopora* species theoretically leads to the sampling of clones, which are genetically identical to their progenitor. Clones are useless samples for the analysis of a population's genetic structure since they are just replicates of independent data points, which thus, cannot be included in the analyses.

In order to avoid sampling clonal colonies, samples are often taken from colonies at least 5m apart from each other (Baums *et al.* 2006; Noreen *et al.* 2009; Souter *et al.* 2009; Combosch and Vollmer 2011). Nevertheless, identical multi-locus genets are identified within the samples of most studies (Ridgway *et al.* 2001 (*P. verrucosa*; 1 m); Baums *et al.* 2006; Souter *et al.* 2009 (*P. verrucosa*; 5 meters distant colonies); Combosch and Vollmer 2011 (*P. damicornis*; samples from 10 meter distant colonies)). In most cases this is interpreted as partially present asexual reproduction among the study case. Strikingly, within the current analysis, not one single identical multi-locus genotype was detected. This leads to the following possible conclusions: (1) *Pocillopora* sp. in the Red Sea exclusively reproduces sexually; (2) By random sampling, clones were not sampled due to their generally low presence or simply by chance, (3) Since this study is based on eight to nine loci, it was possible to disclose conclusion no. 1, which was not possible in previous studies, which were only based on five to six loci.

Conclusions (1) and (3) are not exclusionary and both give a hint on the fact that the reproduction within one species can in fact be solely sexual (not referring to the omnipresent possibility of fragmentation, which would lead to the origin of two or more identical colonies, i.e. clones). Hence, if studies reporting clones within their data set of five to six microsatellite loci would have included more loci, aforementioned conclusion could also have been made (however, not if they were erratically sampling two different species with different reproductive strategies). In other words, simultaneous sexual and asexual reproduction among one single species is most probably unlikely among corals and this fact could most likely have been resolved by previous studies as well if using similarly high molecular resolution.

To sum up, these results shed some light on the confusions over mixed reproductive strategies among *Pocillopora* species and re-launch the widely represented concept of single reproductive strategies for single different species. Previous studies reporting both asexual (clones) and sexual origins of recruits among single species

and/or broadcast spawning as well as brooding of larvae were hence, most likely confounded and mixed sympatric cryptic species in their analysis.

It is also not far fetched to say that an easy way for speciation to occur in the same habitat (sympatric) is for a species to develop different reproductive strategies and so, promote: (1) reproductive isolation and (2) the specialization on a certain niche more appropriate for larvae either produced by sexual or asexual broadcast-spawning or brooding. This reinforces the logic behind not just different timing of gametogenesis but also distinct reproductive mode among sympatric species for the establishment of discrete evolutionary significant units (ESU) and speciation.

5.2.4 Currents and Larval Transport

The mode of reproduction is an important factor in terms of connectivity among isolated sites like separated reefs for corals. Furthermore, in marine environments currents also play a crucial role in terms of genetic dispersal (i.e. gene flow).

On the one hand, currents can represent physical barriers for larval dispersal and thus, gene flow (Lawver *et al.* 1992; Souter *et al.* 2009). On the other, these are also the main and most powerful transport routes for most aquatic larvae (Lessios *et al.* 1999; Treml *et al.* 2008).

There is not much information on current flow patterns in the Red Sea and even less regarding surface water currents. However, it is known that in general, main drivers of surface currents are winds and in the Red Sea a North to South wind is dominant (Fig. 4) (Patzert 1974; Schott and McCreary 2001; Sofianos and Johns 2003, 2007; Chase *et al.* 2006). This might change slightly in strength and direction depending on the monsoon coming from the Gulf of Aden (Sofianos *et al.* 2002; Schott and McCreary 2001; Sofianos and Johns 2007). Nevertheless, those monsoon-related changes have predominantly an effect only in the southern part of the Red Sea (Eshel and Naik 1997; Sofianos and Johns 2007), south of our last sampling site (DOG). Thus, for our study we can assume northern winds driving southward currents along the study sites during the entire year. Such currents would explain connectivity from North to South and not vice-versa. This would speak for the MIGRATE-N results, which are anyhow based on preliminary means (Fig. 3, Tab. 9). However, we observe panmixia along our sampling

sites (omnipotent among all analyses and second most likely model of MIGRATE-N; Fig. 3). Hence, there also is evidently a route for gene flow to the North, in the opposite current-flow direction – but how?

Studies have shown that in some cases water eddies work like baskets transporting larvae/gametes through water bodies (Marshall and Pugh 1996; Bernard et al. 2007). In the Red Sea a permanent cyclonic eddy can be found around 26° N (Sofianos & Johns 2007) (Fig. 4). This eddy might be likely to promote larval dispersal counterclockwise and so, from South to North at the east coast of the Red Sea. Hence, gene flow in both directions is guaranteed, at least for the northern part of the Red Sea – i.e. from KAU to WAJ and vice-versa.

For the southward gamete and larval transport, additional anticyclonic eddies from 17° to 23.5° N (Fig. 4) with velocities of up to 1m/s increase significantly water mixing and promote fast transport (Sofianos and Johns 2007). Since the Red Sea is so narrow (notably near KAU, approx. 200 km width) cyclonic water currents might even be significant enough for the transport of recruits from one shore to the other (from East to West and vice-versa). A permanent anticyclone is found around 23° N with eastward flow around 24° N and western flow at 22° N (Fig. 4). If latter shore-to-shore dispersal holds true, larvae from KAU and DOG (in smaller proportion) driven in anticyclonic direction from North to South at the east coast of the Red Sea could theoretically flow back to North at the west coast again within the eddy. In this manner, not only panmixia between reefs at the east coast (from current investigation) is possible but also beyond these, among reefs on the west coast. In other words, not only the reproductive and larval features of the coral encourage wide genet dispersal but also powerful oceanic water forces increase gene flow over long distance, assisting panmixia within probably most of the Red Sea.

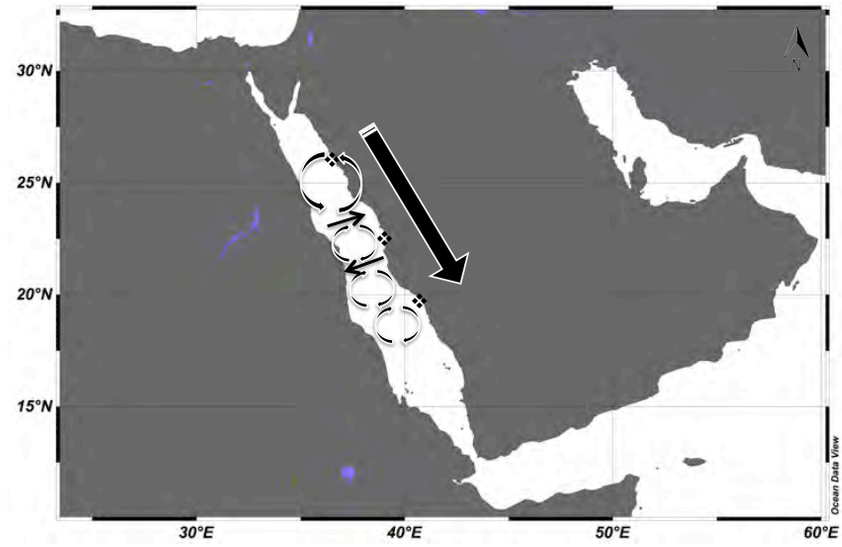

Fig. 4: Wind and Currents in the Red Sea: The biggest arrow stands for the North to South predominant wind direction among the study sites. The northernmost and biggest circle represents a permanent cyclonic eddy. The following circle is also a predominantly permanent eddy with an anticyclonic flow sense. The arrows north (24ºN) and south (23ºN) of it represent the current flow directions produced by this gyre. The two southernmost circles visualize the presence of several anticylonic eddies in that area.

Last but not least, note that DOG is located at 19°N and even though the anticyclonic eddy field ranges to the South up to 17° N, the faster and permanent eddy is north of DOG (at 23° N). This might suggest lower gene flow coming from this site in comparison to that between WAJ and KAU, explaining little but present genic and genetic differentiation found between DOG and KAU. Another reason for aforementioned differentiation, however, could also be related to the T/S gradient examined herein. This will be further discussed in section 5.2.5.

Even though panmixia prevails along the study sites, a consideration of weak genetic structures can give insight on the potential of a population (or a certain reef/site) to cope with changes in the environment and/or to recover after depletion. In the special case of DOG, one should hence, keep in mind that a reduction of this population will possibly have longer effects on its effective size in comparison to WAJ or KAU since it is potentially relatively more isolated from the main gene pool.

5.2.5 Temperature and Salinity Gradient

Pocillopora is one of the most abundant corals in the Pacific and the Red Sea. Species from the Red Sea are also among the reproductively most successful ones in the region (Glassom *et al.* 2004). This is remarkable if one considers their distribution and the fact that they are most plentiful in the upper 5 meters, where the strongest diurnal temperature fluxes are recorded. Additionally, at this upper water layers stress caused by extreme UV penetration as well as temperatures ranging beyond 36º C during daytime on shallow flat reefs are harsh conditions for any other hard coral species (own observation at KAU reefs).

DOG is the only site with hints of genetic isolation (F_{ST}, G_{ST}, D_{EST}, Assignment test). One of the reasons was previously discussed (under 5.2.4). Furthermore, measurements on salinity show an abrupt decrease right north of the DOG sampling site (at 22° N; Sofianos *et al.* 2007). Hence, the described genetic differentiation of DOG might imply a small (not significant) but notable impact of salinity on the success of alien recruits in the area.

In this context, it is predictable to find more genetic differentiation and population structure if analyzing samples from reefs located at the periphery of the gradient and, moreover, of the species distribution. Panmixia among the three study sites can also be due to the temperature and salinity ranges being still within the tolerant limits of the common larvae of *Pocillopora* sp. However, for recruits to successfully settle in the northern- and southernmost regions of the Red Sea, higher adaptation and genetic specialization might be required.

6. Outlook and Perspectives

Based on the preliminary mtDNA analysis, there is evidence for cryptic speciation within single *Pocillopora* morphotypes. In this regard, the application of further genetic markers for an extensive phylogenetic analysis is in preparation.

It would also be interesting to test MSAT primers on the specimens from the putative cryptic species (HP_F) to evaluate population genetic differentiation among ESUs. Hence, an additional comparison of coming results to the current results could be established and give more insight on connectivity of reef building corals of the area and speciation relative to ESUs, reproductive mode and success in recruitment.

In this context, samples from the sister species *Stylophora* sp. (morphotype pistillata) have been taken from the same study sites as *Pocillopora* sp.. *Stylophora* sp., however, reproduces via brooding (Douek *et al.* 2011) whereas *Pocillopora* sp. is a broadcast-spawner. Our working group has developed seventeen MSAT primers for *Stylophora* sp. (Banguera-Hinestroza *et al.* 2013). Such a number of specifically designed and tested markers allows first class population analyses of the Pocilloporidae *Stylophora* sp. Herewith, promising comparisons of related species in same habitats but with different reproductive strategies could be encountered, offering new worthy knowledge on the reefs structure, connectivity, larval dispersal, resilience and health.

Furthermore, we have access to samples from three additional geographic sites for *Pocillopora* and *Stylophora* sp. from which, two are located at the periphery of the gradient. The northernmost would be located at 26° N and the southernmost at 16° N. Further North, it would also be interesting to include samples from the Gulf of Aqaba. Subsequently, an analysis over the entire gradient could be practicable and factors like selective pressure of peripheral populations can also be analyzed in an interspecific comparative manner.

Additionally and as discussed in the sections 5.2.4 and 5.2.5, there is a higher influence of the gradient towards the South of the Red Sea, where additionally incoming waters from the Gulf of Aden change physicochemical as well as biological conditions. This southern region also experiences seasonal changes in the wind direction and herein involved current flow patterns driven by the Indian monsoon system (Patzert 1974; Maillard and Soliman 1986; Schott *et al.* 1990; Van Couwelaar 1997; Schott and McCreary 2001; Sofianos *et al.* 2002).

All in all, this is just the tip of the iceberg and an excellent starting point for further research in the topic, mainly also as there is only very little recent research in this world's marine ecosystem (Berumen *et al.* 2013). As the research continues we expect to provide enough biological and genetical insight in order to soon establish tools for the reefs' conservation. For a good management of the reefs, information on the population's gene flow routes and recruitment sources are of supreme value. One can only know how much gene flow a healthy coral reef requires if populations of its inhabitant species are monitored correctly and we are far from fully understanding the biology and genetic structure of many of these taxa. Thus, assumptions made on the reefs health should be considered in a conservative manner also, because we really do not have pristine reefs left in the oceans, which could serve as a standard control. All we can do is test hypothesis and reject less likely ones, making an approximation to the true state of affairs.

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8. Comments and Notes