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Trans-Pacific RAD-Seq population genomics confirms introgressive hybridization in Eastern Pacific *Pocillopora* corals

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ABSTRACT

Discrepancies between morphology-based taxonomy and phylogenetic systematics are common in Scleractinian corals. In *Pocillopora* corals, nine recently identified genetic lineages disagree fundamentally with the 17 recognized *Pocillopora* species, including 5 major Indo-Pacific reef-builders. *Pocillopora* corals hybridize in the Tropical Eastern Pacific, so it is possible that some of the disagreement between the genetics and taxonomy may be due to introgressive hybridization. Here we used 6769 genome-wide SNPs from Restriction-site Associated DNA Sequencing (RAD-Seq) to conduct phylogenomic comparisons among three common, Indo-Pacific *Pocillopora* species – *P. damicornis*, *P. eydouxi* and *P. elegans* – within and between populations in the Tropical Eastern Pacific (TEP) and the Central Pacific. Genome-wide RAD-Seq comparisons of Central and TEP *Pocillopora* confirm that the morphospecies *P. damicornis*, *P. eydouxi* and *P. elegans* are not monophyletic, but instead fall into three distinct genetic groups. However, hybrid samples shared fixed alleles with their respective parental species and, even without strict monophyly, *P. damicornis* share a common set of 33 species-specific alleles across the Pacific. RAD-Seq data confirm the pattern of one-way introgressive hybridization among TEP *Pocillopora*, suggesting that introgression may play a role in generating shared, polyphyletic lineages among currently recognized *Pocillopora* species. Levels of population differentiation within genetic lineages indicate significantly higher levels of population differentiation in the Tropical Eastern Pacific than in the Central West Pacific.

1. Introduction

The fundamental goal of taxonomy is to arrange taxa in hierarchical categories that reflect their evolutionary or systematic relationships. Taxonomic classifications, however, are still largely based on morphology while evolutionary relationships are commonly described using phylogenetic data (ICZN, 1999; Wiley and Lieberman, 2011). Disagreements between morphology-based taxonomic descriptions and phylogenetic systematics can result from a variety of factors, ranging from phenotypic plasticity to introgressive hybridization (Swofford, 1991). For example, morphological stasis or unexpected convergent evolution of morphological traits (i.e. homoplasy) may result in cryptic species (Bickford et al., 2007), which can be identified with phylogenetic data (e.g. Hebert et al., 2004). However, molecular phylogenies can be misleading as well, for example, due to incomplete lineage sorting and introgressive hybridization (e.g. Arnold, 1997; Pollard et al., 2006).

Introgressive hybridization can confound species' genetic identities by passing genes between taxa. Rates of genetic introgression (i.e. genetic mixing) are often not equal across the genomes of hybridizing species (Vollmer and Palumbi, 2002; Hohenlohe et al., 2013; Larson et al., 2013; Martin et al., 2013; Roux et al., 2013) as genic selection can limit interspecific gene flow of loci involved in hybrid inviability and sterility (speciation genes) while allowing introgression in other parts of the genome (e.g. Martinsen et al., 2001; Wu and Ting, 2004; Brideau et al., 2006). These strongly selected "speciation genes" can define species differences (i.e. genic view of speciation, Wu, 2001) and can be used to study the evolutionary forces that shape species (e.g. Rieseberg et al., 1999, 2003; Presgraves et al., 2003; Savolainen et al., 2006; Via, 2009; Feder et al., 2012; Strasburg et al., 2012). In some cases, introgression can provide evolutionary novelty by passing potentially adaptive genetic variation between species (e.g. Kim and Rieseberg, 1999; Mallet, 2005; Whitney et al., 2010; Kunte et al., 2011; Hedrick, 2013).

Disagreements between morphology- and molecular-based systematics are common in scleractinian corals (e.g. Fukami et al., 2008; Huang et al., 2011). Morphological characters traditionally used in coral taxonomy have been shown to be poor predictors

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of evolutionary relationships, even at the family level (Romano and Palumbi, 1996; Fukami et al., 2004, 2008), and cryptic species are common in many coral genera (e.g. Stobart and Benzie, 1994; Forsman et al., 2009; Keshavmurthy et al., 2013; Boulay et al., 2014). These problems are exacerbated by the fact that mitochondrial markers evolve slowly in corals (e.g. Shearer et al., 2002; Hellberg, 2006) and genetic mixing via introgressive hybridization appears to be common in many genera (reviewed by Willis et al., 2006), which lead to speculation about widespread reticulate evolution among reef corals (Veron, 1995).

Conflicts between molecular phylogenetics and morphology-based species descriptions are common in the Indo-Pacific Scleractinian coral genus *Pocillopora* (Fig. 1) (Combosch et al., 2008; Flot et al., 2010; Souter, 2010; Pinzón and LaJeunesse, 2011; Pinzón et al., 2013; Schmidt-Roach et al., 2013). Lack of genetic monophyly in *Pocillopora* species was first described in the Tropical Eastern Pacific (TEP) and attributed to introgressive hybridization (Combosch et al., 2008). Most TEP *Pocillopora* species share the same rDNA and mtDNA lineage (type 1 after Pinzón et al., 2013; synonymous with "Type III" in Combosch et al., 2008 and "species A" in Flot et al., 2010). However, the most common TEP coral, *P. damicornis*, contains alleles from a second genetic lineage, type 3, which in the TEP is exclusive to *P. damicornis* (Combosch et al., 2008; Fig. 5 in Pinzón and LaJeunesse, 2011). In addition, several *P. damicornis* colonies contain alleles from both lineages, i.e. they are heterozygous for type 1 and type 3 (hereafter T1/3). This suggests a pattern of introgressive hybridization between *P. damicornis* and its TEP congenerics *P. eydouxi* and *P. elegans* that allows for inter-specific gene flow of type 1 alleles into *P. damicornis* (Combosch et al., 2008). *Pocillopora* species with multiple genetic types have subsequently been reported from elsewhere in the TEP (Flot et al., 2010; Pinzón and LaJeunesse, 2011), East Africa (Souter, 2010) and the Great Barrier Reef (Schmidt-Roach et al., 2013). A recent phylogeographic study (Pinzón et al., 2013) identified seven mono-specific lineages and two lineages (types 1 and 3) that were shared by many widespread and well-defined *Pocillopora* morphospecies across broad swaths of the Indo-Pacific. Coral mitochondrial and ribosomal DNA markers (like mtDNA and ITS) have disadvantages for phylogenetic studies in systems with potential hybridization and introgression due to the slow rate of mutation in coral mtDNA markers (e.g. Shearer et al., 2002; Shearer and Coffroth, 2008) and the incomplete concerted evolution of multi-copy coral ribosomal RNAs (Vollmer and Palumbi, 2004). New, next generation sequencing techniques, like RNA-Seq (Chepelev et al., 2009) and RADseq (Baird et al., 2008), should improve phylogenetic analyses of coral species boundaries by providing genome-wide SNP data.

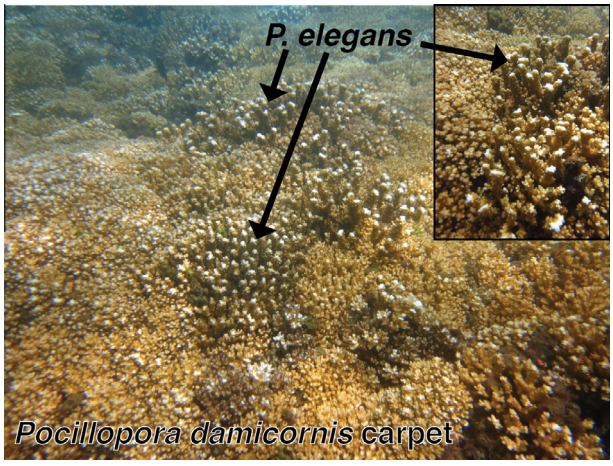

Fig. 1. *Pocillopora elegans* colonies in a *P. damicornis* carpet at Isla Uva, Gulf of Chiriquí, Panamá.

In order to clarify the evolutionary history of the coral genus *Pocillopora*, we applied genome-wide Restriction-site Associated DNA Sequencing (RAD-Seq) to conduct a phylogenomic analysis of three TEP *Pocillopora* species (*P. damicornis*, *P. eydouxi* and *P. elegans*), four trans-Pacific *P. damicornis* populations (from Japan, French Polynesia and two populations from Panamá) and two types of putative TEP hybrids (sensu Combosch et al., 2008). We hypothesized that genome-wide sequence data would confirm previously described genetic lineages. In addition, we expected that the allelic composition of the hybrid samples would confirm that introgressive hybridization is partially responsible for the observed discrepancies with colony morphologies.

2. Material and methods

2.1. Sample collection and DNA extraction

Coral nubbins were collected from *Pocillopora damicornis*, *P. eydouxi* and *P. elegans* in the Gulf of Chiriquí and the Gulf of Panamá (both Panamá, i.e. Tropical Eastern Pacific, TEP) as well as from Central Pacific *P. damicornis* populations in Okinawa (Japan) and the Tuamotu Archipelago (French Polynesia). Morphospecies were identified based on Veron (2000). To identify the different genetic types, 231 specimens were genotyped at the ribosomal internal transcribed spacer 2 (ITS) (Combosch et al., 2008) and a mitochondrial open reading frame (ORF) (Flot and Tillier, 2007), using published protocols. Finally, 147 genotyped specimens were pooled into eight different samples for type-specific RAD-Seq analyses (detailed below; Table 1).

2.2. RAD-Seq

Genomic was extracted using standard protocols and DNA quality was checked using agarose gel electrophoreses after calibration with an Agilent BioAnalyzer 2100 (Agilent Technologies). Eight pooled RAD-Seq samples were generated following Emerson et al. (2010; Table 1). Briefly, DNA from Tropical Eastern Pacific *P. damicornis* specimens with pure ITS type 3 genotypes was pooled into two population samples for Gulf of Chiriquí specimens ("*P. damicornis*-T3-Panama1") and Gulf of Panamá specimens ("*P. damicornis*-T3-Panama2"). Species-specific samples were also generated for the Tropical Eastern Pacific conspecifics *P. eydouxi* and *P. elegans*, containing pure ITS type 1 genotypes, by pooling DNA from Panamanian specimens in the samples "*P. eydouxi*" and "*P. elegans*". These four samples represent the parental species in the TEP hybridization system and were compared with two samples containing potential hybrid specimens. Namely, TEP *P. damicornis* specimens with heterozygous ITS type 1 and type 3 genotypes were pooled in the sample "*P. damicornis*-T1/3-Panama" and *P. damicornis* specimens with the conspecifics ITS genotype type 1 were pooled in the sample "*P. damicornis*-T1-Panama". In addition, Central Pacific *P. damicornis* specimens with ITS type 5 genotypes from Japan and the Tuamotus were pooled in the samples "*P. damicornis*-T5-Japan" and "*P. damicornis*-T5-Tuamotus", respectively, to serve as outgroup for TEP comparisons and enable comparisons between trans-Pacific *P. damicornis* populations.

RAD-Seq libraries were prepared following modified published protocols (Etter et al., 2011). Genomic DNA was digested using high-fidelity *Pst1* (New England Biolabs). Resulting fragments were ligated to Pst1-P1 adapters that contained sample-specific barcodes and primer annealing sites. These DNA constructs were randomly fragmented to an average size of 400 bp, using a Covaris S220 Ultrasonicator (Covaris Inc.) and 300–500 bp fragments were

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Table 1
Overview of the RAD-Seq sample composition and basic population genetic indices. Central-west Pacific *P. damicornis* populations were characterized by high levels of genetic polymorphism (9–12%), many private alleles (>800) and high proportions of fixed private alleles (5–10%). Most TEP samples (*P. eydouxi*, *P. elegans*, *P. damicornis*-T1-Panama and *P. damicornis*-T1/3-Panama) had low levels of polymorphisms (7–8%), few private alleles (<250) and low proportions of fixed private alleles (<2%). No significant correlations were found between the number of raw reads and the number of pooled individuals per sample (Fig. S1) with the number of heterozygous loci and/or the number of private alleles.

Sample	Region	Location	Genotypes ITS2	Genotypes ORF	N_{pool}	Reads [*M*]	Variable loci	Het loci	PA[a]
P. damicornis T5-Japan	CWP	Japan	Type 5	Type 5	17	3.5	18,350	**1405**	**842**
P. damicornis T5-Tuamotus	CWP	Tuamotus	Type 5	Type5	23	9.3	26,210	**1793**	**993**
P. damicornis T3-Panama1	TEP	Gulf of Chiriquí	Type 3	Type 3	21	17.6	27,037	1151	**539**
P. damicornis T3-Panama2	TEP	Gulf of Panamá	Type 3	Type 3	18	7.6	21,533	1561	240
P. damicornis T1/3-Panama	TEP	Gulf of Chiriquí & Gulf of Panamá	**Type 1/3**	Type 1	21	26.3	31,586	1217	135
P. damicornis T1-Panama	TEP	Gulf of Chiriquí & Gulf of Panamá	Type 1	Type 1	13	9.5	15,275	1201	116
P. eydouxi	TEP	Gulf of Chiriquí & Gulf of Panamá	Type 1	Type 1	16	22.5	33,301	1224	225
P. elegans	TEP	Gulf of Chiriquí & Gulf of Panamá	Type 1	Type 1	18	2.8	29,293	1074	167
					147	**99.1**	**39,581**	**6128**	**3242**

Region: CWP = Central-West Pacific; TEP = Tropical Eastern Pacific; Location: Tuamotus = French Polynesia; Gulf of Chiriquí & Gulf of Panamá = Panamá; N_{pool} = Number of pooled specimens; Reads [*M*] = number of raw reads [in million reads] after quality control; Het loci = heterozygous loci = loci that are variable within samples.
[a] PA = Private Alleles.

size-selected using agarose gels. DNA fragments were ligated to a second adapter (P2) and PCR-amplified using Phusion Polymerase, and a PCR cycle with 14–18 cycles at 98 °C for 10 s, 65 °C for 30 s and 72 °C for 30s with an initial denaturation step at 98 °C for 30 s and a final extension step at 72 °C for 5 min. Resulting libraries were size-selected (350–550 bp) to remove remaining adapters and primers and quantified on an Agilent BioAnalyzer 2100 (Agilent Technologies) and a Qubit Fluorometer (Invitrogen). Multiplexed samples were single-end sequenced (1×50 bp, 4 samples per lane) on an Illumina HiSeq 2000 at the Center for Systems Biology (Harvard University).

Quality filtering and loci assembly was conducted using STACKS v0.999 (Catchen et al., 2013). Samples were de-multiplexed and raw reads were quality trimmed to remove reads with low-quality (Phred score <30), more than one ambiguous nucleotide or primer sequences, and reads without complete barcode or restriction cut site. Barcode sequences were trimmed and the 47 bp reads were assembled into 'stacks' (the RAD-Seq equivalent of alleles) if five or more identical reads were found within a sample ($m = 5$). Invariant stacks were then compiled into sample-specific loci if they differed by less than 2 nucleotides (i.e. ~5% divergence; $M = 2$). Sample-specific loci were then assembled into homologous loci if they differed by less than 3 nucleotides (~7%, $n = 3$) between samples. Assembly parameters (m 5; M 2; n 3) were chosen after empirical testing (m: 3–20; M: 1–3; n: 1–3) and subsequent loci validation with *P. damicornis* transcriptomes (detailed below). The applied parameter setting resulted in the highest number and proportion of loci aligning to unique locations of the transcriptome (Table S2).

Single nucleotide polymorphisms (SNP) were identified with maximum likelihood models, implemented in STACKS. In addition, three restrictive filters were applied to increase the proportion of unique genomic loci: (1) all loci with more than 1 SNP were removed (due to the short length of sequence reads), (2) the STACKS deleveraging algorithm was used to identify and remove significantly highly repetitive stacks that likely represent sequencing errors and/or repetitive genomic regions, and (3) the STACKS populations program was used to filter out loci with invalid SNP calls. The remaining RAD loci consist of 47 bp of sequence data per locus with up to one nucleotide position that varied within and/or across samples.

To identify RAD loci that resulted from protein-coding gene regions, a TEP *P. damicornis* reference transcriptome was assembled de novo from RNA-Seq data. For the reference transcriptome, four Panamanian *P. damicornis* specimens were mRNA-sequenced to generate a TEP-specific transcriptome (Table S2). Detailed material and methods for the RNA-Seq data generation and de novo transcriptome assembly are included in the supplementary materials. Transcriptome-mapped RAD loci were used to identify coral versus symbiont loci and annotate protein-coding RAD loci. For each RAD locus, a strict majority consensus sequence was generated and aligned to the de novo transcriptome using Bowtie 0.12.9 with up to one mismatch. In addition, RAD loci were also aligned to a publicly available *P. damicornis* transcriptome (Traylor-Knowles et al., 2011) and transcriptomes of two different *Symbiodinium* types (C and D; Ladner et al., 2012) that are commonly found in TEP and Central West Pacific *Pocillopora* (e.g. Cunning et al., 2013). Putative gene identities were determined using homology searches with BLASTx against the Swiss-Prot protein database. Blast matches with an *e*-value of less than 10^{-5} were deemed homologous and their GO terms and gene functions were obtained using Blast2Go.

2.3. RAD-Seq analyses

The use of pooled DNA samples for RAD-Seq provides an efficient and economical way to investigate average allele frequency differences between defined groups at many loci. Pooled RAD-Seq cannot reveal genotypic information per sample. Our phylogenetic and population genetic analyses therefore depend on the most frequent alleles in each sample pool. Assembled RAD loci were organized in two datasets: All loci that were recovered from at least two samples were compiled in the extensive 'P2' dataset, which was used for all analyses, unless stated otherwise. Loci that were found in all 8 samples were assembled in the more conservative 'P8' dataset, which was used to calculate pairwise population genetic metrics (e.g. heterozygosity, private alleles, pairwise distances) and to verify and support P2 results. In addition, reduced P2 and P8 datasets containing only homozygous loci (i.e. fixed within samples but variable between) were compiled for phylogenomic analyses as in Emerson et al. (2010). Phylogenomic alignments contained a single consensus sequence per sample consisting of concatenated RAD loci, using IUPAC ambiguity base calls to code for heterozygous SNPs.

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Maximum Likelihood (ML) analyses were conducted with RAxML 8.0 (Stamatakis, 2014) on the CIPRES Web Portal (Miller et al., 2010). We used the GTR-GAMMA model, which includes a parameter for site heterogeneity, and 1000 rapid bootstrap replicates to estimate clade confidence. Maximum parsimony (MP) analyses were used to validate ML results using the program dnapars (Phylip version 3.695; Felsenstein, 2005) with 111 randomized sample input orders (using the "jumble" option) and default settings ('More thorough search'; 'Ordinary Parsimony'; 'retaining 10,000 trees while searching'). Bootstrap support was assessed with 500 (P2) and 1000 (P8) bootstrap replicate datasets, respectively, that were generated with the program seqboot (Phylip 3.695).

Heterozygote loci (i.e. loci that are polymorphic within populations), private alleles and pairwise comparisons were calculated using parsing scripts in R (R Core Team, 2012). Pairwise genetic distances between samples were calculated in three different ways. The most conservative approach (P-distance) is based on the proportion of differentially fixed loci over all fixed loci in each pairwise comparison between samples (Nei and Kumar, 2000). P-distances were visualized using Principal Coordinate Analyses (PCoA) as implemented in GenAlEx (Version 6.4, Peakall and Smouse, 2006). To include heterozygous loci, two pairwise distance approaches, developed for microsatellite (Bowcock et al., 1994) and SNP data (Isolation-by-state, IbS), were tested. The microsatellite method is based on the proportion of shared alleles in each pairwise comparison (e.g. A/A versus A/C = ½). The IbS method is based on the proportion of identical allele comparisons (i.e. A/A versus A/C = ¾). Since both methods resulted in virtually identical pairwise distances, only the IbS results are presented here. Both P- and IbS-distances are reported as proportional differences (Table 3; Table S3 shows the total number of differently fixed loci and allelic differences). To assess genome-wide levels of selection among samples, pairwise distances between samples were compared at transcribed and un-transcribed loci (Fig. S2).

To determine the degree of genetic admixture, patterns of allele sharing in putative hybrid samples (*P. damicornis*-T1/3-Panama and *P. damicornis*-T1-Panama) were analyzed at 4333 loci segregating between type 1 *P. eydouxi* and *P. elegans* versus type 3 *P. damicornis* from the Gulf of Chiriquí (*P. damicornis*-T3-Panama1).

3. Results

3.1. RAD-Seq loci

Over 154 million high-quality RAD-Seq reads were obtained with an average of 12.4 million reads per pooled sample. STACKS assembled 207,228 RAD loci that were shared between two or more samples and 33,710 loci (7.5%) that were found in all eight samples. Quality filtering reduced these numbers to 15,511 P8 loci with 6769 single nucleotide polymorphisms (SNPs) from all eight samples (average cover 133×) and 112,644 P2 loci with 39,581 SNPs from at least two samples (average cover 89×; Table S1). Our main RAD-based phylogenomic alignment covered 1,860,307 bp genome-wide sequence data, including 39,581 SNPs.

3.2. RAD-Seq transcriptome matches

The assembled RNA-Seq transcriptome for TEP *P. damicornis* consisted of 72,522 contigs with an average length of 510 nucleotides and a N50 of 628 nucleotides (Combosch and Vollmer, in prep). Approximately one quarter of all RAD P8 loci (3831 out of 15,511) mapped to the new transcriptome and/or the published *P. damicornis* transcriptome (Traylor-Knowles et al., 2011) and 10% of the 73,522 transcriptome contigs contained a RAD locus (Table S2). In contrast, less than 0.001% of all RAD loci ($n = 35$ and 42, respectively) mapped to the publicly available *Symbiodinium* transcriptomes (types C and D; Ladner et al., 2012) (Table S2). This indicates that the RAD-Seq data/loci are predominantly coral.

3.3. RAD-Seq analyses

RAD phylogenomic analyses consistently returned three well-supported clades, regardless of dataset or phylogenetic method (Fig. 2), which matched the three genetic groups (types 1, 3 and 5) previously identified by ITS and ORF sequencing (Table 1) (Pinzón et al., 2013). Clade 1 contained the two TEP morphospecies *P. eydouxi* and *P. elegans* and both hybrid samples (*P. damicornis*-T1-Panama and *P. damicornis*-T1/3-Panama). Clade 3 contained both TEP *P. damicornis* populations (*P. damicornis*-T3-Panama1 and 2). Clade 5 contained both Central West Pacific *P. damicornis* populations from Japan and the Tuamotus. *Pocillopora damicornis* populations in the TEP (clade 3) and in the Central West Pacific (clade 5) were genetically distinct, separated by significant phylogenetic distances with 100% bootstrap support while phylogenetic relationships within clade 1 were not well supported (Figs. 2 and S2).

Pairwise comparisons confirmed large genetic differences between the three clades (Fig. 3, Tables 2 and S3). Less than 5% of fixed SNPs differed within clades while over 20% differed between clades. Notably, *P. damicornis* from the Gulf of Panamá (*P. damicornis*-T3-Panama2) shared more alleles with its type 1 congeners *P. elegans* and *P. eydouxi* than its *P. damicornis* counterpart from the Gulf of Chiriquí (*P. damicornis*-T3-Panama1; Fig. 3).

Within clades, pairwise comparisons indicated higher levels of genetic differentiation between geographic populations than between morphospecies as currently described. For example, the two Central Pacific type 5 populations and the two TEP type 3 populations showed comparable levels of population structure. Interestingly, the Central West Pacific populations are separated by more than 10,000 km while the TEP populations show the same level of differentiation over a much shorter geographic distance (~400 km). However, the intermediate genetic identity of the *P. damicornis* sample from the Gulf of Panama (*P. damicornis*-T3-Panama2; Fig. 3) indicates that the RAD comparison might be affected by admixture (introgression) from type 1 lineages. In contrast, pairwise comparisons revealed little evidence for genetic differentiation among type 1 samples, including *P. eydouxi*, *P. elegans* and the *P. damicornis* hybrids (*P. damicornis*-T1-Panama and *P. damicornis*-T1/3-Panama). The morphospecies *P. eydouxi* and *P. elegans* differed by 12 fixed SNPs (P-distance: 0.003; the largest within this group) while the two hybrid pools did not differ by a single fixed SNP. Remarkably, the heterozygous *P. damicornis* sample (*P. damicornis*-T1/3-Panama) was less different from *P. damicornis* samples in clade 3 and 5 than other type 1 samples (Fig. 3, Table 2).

3.4. Evidence for introgressive hybridization

The genetic make-up of the two putative *P. damicornis* hybrid pools – *P. damicornis*-T1/3-Panama and *P. damicornis*-T1-Panama – was analyzed in detail to detect signatures of genetic admixture at the 4,333 segregating (i.e. fixed) loci between the "parental" type 3 (*P. damicornis*-T3-Panama1) and type 1 lineages (*P. eydouxi* and *P. elegans*; Table 3). Both *P. damicornis* hybrid pools were dominated by type 1 alleles. Heterozygous ITS type 1 and 3 specimens (*P. damicornis*-T1/3-Panama) contain 87% of type 1 alleles and 13% type 3 alleles at segregating loci with 78% of segregating loci fixed for Type 1, 18% heterozygous and 4% fixed for type 3. *Pocillopora damicornis* specimens homozygous for ITS type 1 (i.e. the *P. eydouxi* and *P. elegans* type) contained 94% type 1 alleles at

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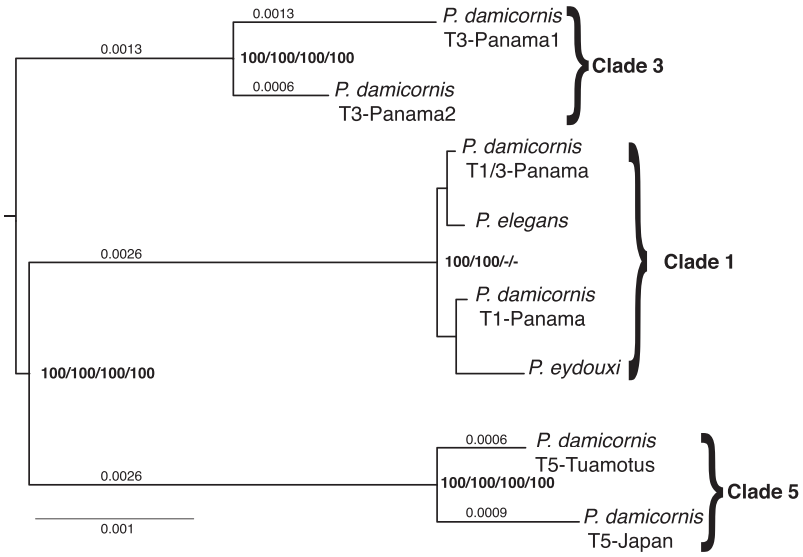

Fig. 2. RAD phylogenomic analyses. Tree topology and branch lengths [substitutions/site] are based on Maximum likelihood reconstructions (RAxML). Bootstrap support (in bold) is based on 1000 pseudo-replicates for RAxML and 500 pseudo-replicates for Maximum Parsimony (DNApars) and reported for analyses based on all loci and fixed SNPs loci only (RAxML/fix RAxML/DNApars/fix DNApars). The tree topology was identical among all four analyses with the exception of one node (intra type 1) in the fixed Maximum Parsimony analyses. The tree topology for P8 loci was identical and the bootstrap support was virtually identical (results not shown).

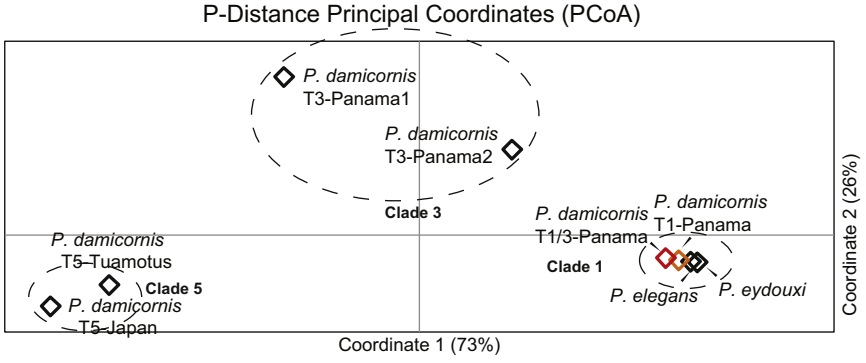

Fig. 3. Principal Coordinate Analysis of pairwise P-distances between RAD-Seq samples. PCA is drawn proportionally (i.e. according to percentages per axis). The first principal component explains 73% of the variance (53% for IBS, results not shown) and separated the pure *P. damicornis* samples from the hybridizing samples in clade 1. The second principal component explained 26% of the genetic variance and separates the clade 3 from the others. Among type 1 sample, the two hybrids (*P. damicornis*-T1/3-Panama ◇ and *P. damicornis*-T1-Panama ◇) are more similar to other *P. damicornis* samples than the other two type 1 samples (*P. eydouxi* and *P. elegans*). The unexpected intermediate identity of *P. damicornis*-T3-Panama2 is also evident.

segregating loci and 6% type 3 alleles with 89% of segregating loci fixed for type 1, 10% heterozygous and 1% fixed for type 3. Remarkably, 21 out of the 34 loci that were fixed for type 3 alleles in homozygous hybrids were also fixed for type 3 alleles in heterozygous hybrids, which suggests that these fixed loci are not a random sample of the 4,333 segregating loci but a selected subset of *P. damicornis* specific alleles ($Chi^2 = 378$, $p < 0.001$, d.f. = 1).

One hundred five (105) loci segregated between TEP samples with *P. damicornis* morphologies and samples with *P. eydouxi* and/or *P. elegans* morphologies. Preliminary analyses against our transcriptome indicated that 10 of these fixed RAD loci map to the transcriptome. Three of these contigs have well-defined protein annotations (*e*-value 10^{-5}) and all three corresponding proteins are all involved in gene regulation (SND1, FBP1 and RBM19). Regulatory genes are prime candidates for hybrid sterility and inviability, i.e. speciation genes (e.g. Wu and Ting, 2004; Ortíz-Barrientos et al., 2007), and several speciation genes in other taxa were found to have regulatory functions (Ting et al., 1998; e.g. Ods & Hmr; Brideau et al., 2006). A trans-Pacific comparison of the 105 segregating loci revealed that 33 of these loci were fixed for *P. damicornis* alleles in *P. damicornis* samples from Japan and the Tuamotus, i.e. across all three genetic lineages and over 20,000 km of open ocean. The remaining 72 segregating loci were either absent ($n = 56$) or fixed for *P. eydouxi*/*P. elegans*-alleles ($n = 16$); only 5% were mixed within or between samples (compared to 35% genome-wide). This pattern suggests that these fixed *P. damicornis* loci are suitable candidate "speciation genes" to further explore genetic differences between *Pocillopora* morphospecies, within and across genetic lineages.

4. Discussion

By characterizing the evolutionary relationships among three common *Pocillopora* morphospecies and their putative TEP hybrids using RAD-Seq, we confirm *P. damicornis*, *P. eydouxi* and *P. elegans* are not monophyletic, but are instead comprised of multiple genetic lineages that are shared among morphospecies. The morphospecies *P. damicornis* possesses three genetic lineages (types 1, 3 and 5). *Pocillopora damicornis* from the CWP, which also differ in their reproductive strategies (Torda et al., 2013), contain a different lineage (type 5) than their TEP counterparts (types 1 and

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Table 2
Pairwise comparisons between samples using P-distance and IbS-proportions. The average amount of differently fixed SNPs between sample pairs was 588; since 4187 loci were on average fixed between samples, the average P-Distance was 0.14. Allele-specific pairwise comparisons (IBS) indicated on average 7073 alleles differences in 27,076 allelic comparisons, which correspond to an average IbS of 1780 and an average IbS-proportion of 0.26. The average sample pair was thus fixed for the same allele at 12,901 P8 loci, for different alleles at 588 loci and contained 2000 heterozygous loci.

	P. damicornis T5-Japan	*P. damicornis* T5-Tuamotus	*P. damicornis* T3-Panama1	*P. damicornis* T3-Panama2	*P. damicornis* T1/3-Panama	*P. damicornis* T1-Panama	*P. elegans*	*P. eydouxi*
P. damicornis T5-Japan		0.227	0.313	0.324	0.333	0.336	0.334	0.341
P. damicornis T5-Tuamotus	0.045		0.315	0.327	0.335	0.338	0.337	0.344
P. damicornis T3-Panama1	0.206	0.183		0.196	0.302	0.306	0.305	0.312
P. damicornis T3-Panama2	0.198	0.176	0.046		0.237	0.239	0.238	0.244
P. damicornis T1/3-Panama	0.233	0.212	0.204	0.091		0.116	0.120	0.127
P. damicornis T1-Panama	0.238	0.218	0.210	0.094	0.000		0.117	0.124
P. elegans	0.244	0.224	0.214	0.100	0.001	0.001		0.125
P. eydouxi	0.246	0.227	0.218	0.099	<0.001	0.001	0.003	

Above: IbS-proportions = Proportion of allele comparisons that differ between samples; Average = 0.261 (±0.094).
Below: P-distance = Proportion of fixed loci that differ between samples; Average = 0.140 (±0.084).
Intra-clade comparisons are boxed in.
Grey backgrounds highlight the increasing pairwise distances between type 1 samples and *P. damicornis* samples with ITS type 3 and type 5 (*P. damicornis*-T1/3-Panama < *P. damicornis*-T1-Panama < *P. eydouxi*/*P. elegans*).

Table 3
Genetic make-up of putative hybrid *P. damicornis* samples. Genetic make-up of putative hybrid *P. damicornis* samples (*P. damicornis*-T1/3-Panama and *P. damicornis*-T1-Panama) at 4333 loci that are segregating between type 3 (*P. damicornis*-T3-Panama1) and type 1 (*P. eydouxi*/*P. elegans*). Percentages are calculated based on the number of alleles/loci present in each sample. 581 segregating loci were not recovered from *P. damicornis*-T1/3-Panama, 882 loci were not recovered from *P. damicornis*-T1-Panama and 1065 were not recovered from either one (i.e. "Both").

	Alleles				Loci					
	Type 3 (*P. damicornis* T3-Panama1)		Type 1 (*P. eydouxi*/ *P. elegans*)		Type 3 (*P. damicornis* T3-Panama1)		Type 1/3 mixed		Type 1 (*P. eydouxi*/ *P. elegans*)	
Type 3 (*P. damicornis* T3-Panama1)	8666	100%			4333	100%				
P. damicornis T1/3-Panama	**967**	**13%**	**6630**	**87%**	**134**	**4%**	**699**	**18%**	**2962**	**78%**
P. damicornis T1-Panama	**403**	**6%**	**6494**	**94%**	**34**	**1%**	**335**	**10%**	**3077**	**89%**
Both Hybrids	298	5%	5850	90%	21	1%	230	7%	2638	81%
Type 1 (*P. eydouxi*/*P. elegans*)			8666	100%					4333	100%

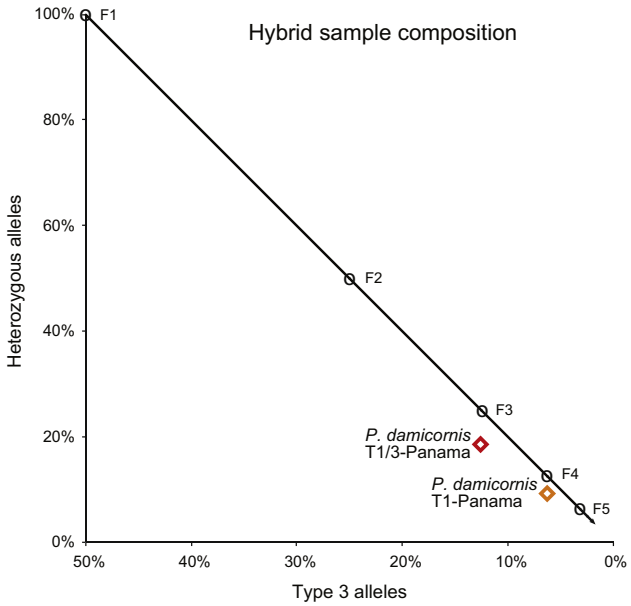
To evaluate levels of random 'background' variation of SNPs between lineages, *P. eydouxi* and *P. elegans* were analyzed vice versa at all 3,723 loci segregating between type 3 (*P. damicornis*-T3-Panama1) and the two hybrids (*P. damicornis*-T1/3-Panama and *P. damicornis*-T1-Panama) (Table S4a).

3, respectively) (Combosch et al., 2008). TEP *P. eydouxi* and *P. elegans* share the common genetic lineage type 1 (Combosch et al., 2008; Pinzón and LaJeunesse, 2011) and differed by only 0.3% (P-distance, Table 2), indicating that they are closely related. Interestingly, genome-wide differences between two sampled TEP populations of *P. damicornis* type 3, separated by 400 km, far exceeded the observed levels of population differentiation within Central Pacific *P. damicornis* (type 5) over much larger geographic distances (e.g. the 11,400 km separating Japan and French Polynesia; Table 2).

4.1. Hybridization

RAD-Seq comparisons between putative hybrid and pure-type *P. damicornis* samples revealed patterns of genetic mixing consistent with introgressive hybridization between the type 1 and 3 lineages in Panamá, and identified a subset of fixed (i.e. species-specific) RAD alleles at 105 loci that are shared among *P. damicornis* specimens, regardless of their introgression history and their geographic origin. Evidence for introgressive hybridization between Tropical Eastern Pacific (TEP) *Pocillopora* species so far included the presence of heterozygous ITS type 1/3 genotypes in Panamanian *P. damicornis* specimens (Combosch et al., 2008), admixture signatures in multi-locus microsatellite data (Pinzón et al., 2013) and mismatching ITS and ORF genotypes (detailed below). The RAD-Seq data revealed that *P. damicornis* heterozygous for ITS type 1 and 3 (*P. damicornis*-T1/3-Panama) are genetically closer to other TEP type 1 samples (*P. elegans* and *P. eydouxi*) than to pure *P. damicornis* type 3 (*P. damicornis*-T3-Panama1; Fig. 3). This is expected if heterozygous specimens are the result of hybridization between types 1 and 3, but cannot be explained as a result of ITS incomplete lineage sorting in type 3 (as proposed by Pinzón and LaJeunesse, 2011).

The presence of type 1 and type 3 alleles in both homozygous and heterozygous states in both hybrid samples (Table 3) suggests that the group of sampled hybrid *P. damicornis* specimens were at least a few generations removed from their most recent type 3 ancestors (Fig. 4). As indicated by their ITS genotypes, the group



Backcross Generation	F1	F2	F3	*Pdam*_T1/3	F4	*Pdam*_T1	F5
Heterozygous alleles	100%	50%	25%	**18%**	13%	**10%**	6%
Type 3 alleles	50%	25%	13%	**13%**	6%	**6%**	3%

Fig. 4. Allele composition of putative hybrid *P. damicornis* samples compared to the expected allele compositions of backcrossing hybrids over several generations. The heterozygote sample *P. damicornis*-T1/3-Panama (◇) are is on average only 3–4 generations removed from the initial hybridization event while *P. damicornis*-T1-Panama (◇) is on average 4–5 generations removed.

of homozygous ITS hybrids (*P. damicornis*-T1-Panama) are further removed from their most recent type 3 ancestors than heterozygous ITS hybrids (*P. damicornis*-T1/3-Panama; Fig. 4). Backcrossing and hybridization in general seems dominated by type 1 maternal lineages (and eggs) since virtually all heterozygote samples had mitochondrial type 1 genotypes. Since these colonies have *P. damicornis* morphologies, maternal inheritance does not seem to dominate colony morphology as in Caribbean *Acropora prolifera* hybrids (Vollmer and Palumbi, 2002).

The dominant species of TEP coral reefs, *P. damicornis*, is the central component of the local hybridization system. It is the only species in the TEP that frequently contains both genetic lineages and TEP populations are the only ones that contain genotypes heterozygous for ITS type 1 and 3. Out of the 191 *P. damicornis* sampled from Panama here and by Combosch and Vollmer (2011), 40% are homozygous for type 3, 14% are heterozygous (type 1/3), and 46% are homozygous for type 1. Despite population genetic evidence for significant regional differentiation of *P. damicornis* populations between the upwelling Gulf of Chiriquí and the non-upwelling Gulf of Panamá (Combosch and Vollmer, 2011), we detected no significant difference in the geographic distribution of hybrid versus pure-type specimens.

Moreover, not a single ITS type 3 genotype has so far been found anywhere else in the TEP, despite extensive genotyping in Mexico, Galapagos and Clipperton (~300 genotyped samples, Flot et al., 2010; Pinzón and LaJeunesse, 2011). Since type 3 genotypes are widespread throughout the vast Indo-Pacific, dispersal barriers are unlikely to account for the geographic limitation of type 3 genotypes in the TEP. Selection against type 3 genotypes might prevent these genotypes from establishing a significant presence outside of Panama, even in the absence of geographic and/or reproductive barriers (isolation-by-ecology, e.g. Prada and Hellberg, 2013). Since none of the surveyed Mexican reefs are located in an upwelling zone, the more heterogeneous environment in Panamá may benefit the sympatric coexistence of both genotypes. Differences in the thermal regimes in upwelling and non-upwelling zones in Panama have been shown to effect the performance of different *P. damicornis* genotypes (D'Croz and Mate, 2004) and may explain why type 3 are not found elsewhere in the TEP.

Evidence for introgressive hybridization among *Pocillopora* species and lineages have been reported from several other Indo-Pacific locations (e.g. Miller and Ayre, 2004; Souter, 2010; Pinzón et al., 2013; Schmidt-Roach et al., 2013). Moreover, re-analyzes of published sequence data (Flot et al., 2008a) revealed heterozygote ITS genotypes and mismatching ITS and ORF genotypes in 70% of Hawaiian *P. damicornis* specimens (11 out of 16), including a single specimen with alleles from three distinct genetic lineages (heterozygote ITS type 3/5 and ORF type 4), as well as mismatching ITS and ORF genotypes in seven *P. meandrina* and one *P. eydouxi* specimens. These mixed-type individuals strongly suggest that introgressive hybridization may be common in Hawaiian *Pocillopora*.

4.2. Colony morphology, phylogenetics and the evolution of *Pocillopora* corals

The main result of this study, that rDNA- and mtDNA-based genetic types are consistent with genome-wide RAD-Seq lineages, strongly supports the hypothesis that some of the genetic types described in *Pocillopora* corals correspond to distinct, reproductively isolated species (sensu Mayr, 1963). Additional morphological, ecological and reproductive studies are still necessary to identify and delimitate valid *Pocillopora* species within and among genetic lineages. Importantly, genetic types 1, 3 and 5 analyzed here correspond to the three major lineages in *Pocillopora* (Pinzón et al., 2013). Several other *Pocillopora* types (or lineages) have low phylogenetic support (e.g. types 4, 7 and 9) or are found in only a few individuals (type 8).

The lack of monophyly in the currently defined *Pocillopora* species indicates a disconnect between colony morphology and genetic lineage (or type). If *Pocillopora* species were defined solely by genetics, species would be comprised of multiple distinct colony morphologies. Colony morphologies can be shared because they are ancestral, convergent and/or plastic (i.e. taxonomy is based on unsuitable characters), or because the underlying "morphology genes" moved across species boundaries via introgressive hybridization. Highly variable, plastic morphologies are widespread among *Pocillopora* corals, but are unlikely to alone account for the range of discrepancies observed here, including eight distinct, recurrent colony morphologies in two distinct genetic lineages (Pinzón et al., 2013). Phenotypic plasticity depends on environmental differences to drive morphologic variation (West-Eberhard, 1989) and many of these morphospecies occur next to each other, i.e. sympatrically (Fig. 1). Moreover, studies have shown consistent links between *Pocillopora* morphospecies and specific genotypes (e.g. Schmidt-Roach et al., 2013). Divergent selection can generate and/or maintain genetically determined recurrent colony morphologies within distinct genetic lineages via convergent evolution, morphological stasis or introgressive hybridization. However, none of them can account for all eight observed morphospecies shared between type 1 and type 3. In turn, none of these scenarios are mutually exclusive and some of them are in fact more likely to occur together than separately, for example due to mutually favorable conditions (e.g. strong selection). It is likely that a combination of factors generates the observed discrepancies in *Pocillopora* corals.

In the TEP, disagreements between colony morphology and phylogenetics can be explained by introgressive hybridization as shown here and in several other studies (e.g. Combosch et al., 2008; Pinzón and LaJeunesse, 2011). It is unlikely, however, that

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introgressive hybridization explains all disagreements between morphological taxonomy and phylogenetic lineages throughout the Indo-Pacific. It is a first step, however, and we have shown that the isolated Tropical Eastern Pacific, with its reduced complexity of *Pocillopora* corals, is a good place to disentangle *Pocillopora* species' boundaries.

Significant discrepancies between morphology-based taxonomy and phylogenetic systematics are common in other pocilloporiid genera and have been described in *Stylophora* (Flot et al., 2011; Stefani et al., 2011), *Seriatopora* (Flot et al., 2008b) and *Madracis* (Frade et al., 2010). Different evolutionary scenarios and combinations of mechanisms have been proposed for each genus. For example, while introgressive hybridization has been documented in *Madracis* (Frade et al., 2010) and *Pocillopora* (Combosch et al., 2008; Schmidt-Roach et al., 2013; this study), it has been dismissed in *Stylophora* based on the congruence of nuclear and mitochondrial markers (Flot et al., 2008b). Studies in all four genera have benefitted from common transferable technical advances. For example the description of the mitochondrial genome in *Pocillopora* (Flot and Tillier, 2007) enabled the development of mitochondrial sequence markers in *Seriatopora* (Flot et al., 2008b) and *Stylophora* (Flot et al., 2011). We hope that his study testifies to the future utility of genome-wide RAD sequencing to disentangle the complex evolutionary history of corals in general and pocilloporiids in particular.

5. Conclusions

Genome-wide RAD data supports the lack of monophyly among well-recognized *Pocillopora* species. Additional genetic, morphological, ecological, and reproductive studies will be necessary (e.g. Combosch and Vollmer, 2013; Torda et al., 2013) to resolve these conflicts in *Pocillopora* taxonomy in light of the genetic findings published here and elsewhere. Several recent publications have made significant progress (e.g. Stefani et al., 2008; Benzoni et al., 2010; Arrigoni et al., 2012; Schmidt-Roach et al., 2013). Further phylogenetic analyses must improve our evolutionary understanding of these taxa, resolve the taxonomy, and provide field biologists with a means to identify these ecological important corals species in the field.

Data accessibility

RAD-Seq raw reads and P2 and P8 loci alignments are available from the Dryad data repository (Combosch & Vollmer, 2015; http://dx.doi.org/10.5061/dryad.436h0).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.022.

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