MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 311–325 doi: 10.1111/j.1365-294X.2010.04939.x

Species delimitation of common reef corals in the genus *Pocillopora* using nucleotide sequence phylogenies, population genetics and symbiosis ecology

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Abstract

Stony corals in the genus *Pocillopora* are among the most common and widely distributed of Indo-Pacific corals and, as such, are often the subject of physiological and ecological research. In the far Tropical Eastern Pacific (TEP), they are major constituents of shallow coral communities, exhibiting considerable variability in colony shape and branch morphology and marked differences in response to thermal stress. Numerous intermediates occur between morphospecies that may relate to extensive hybridization. The diversity of the *Pocillopora* genus in the TEP was analysed genetically using nuclear ribosomal (ITS2) and mitochondrial (ORF) sequences, and population genetic markers (seven microsatellite loci). The resident dinoflagellate endosymbiont (*Symbiodinium* sp.) in each sample was also characterized using sequences of the internal transcribed spacer 2 (ITS2) rDNA and the noncoding region of the chloroplast psbA minicircle. From these analyses, three symbiotically distinct, reproductively isolated, nonhybridizing, evolutionarily divergent animal lineages were identified. Designated types 1, 2 and 3, these groupings were incongruent with traditional morphospecies classification. Type 1 was abundant and widespread throughout the TEP; type 2 was restricted to the Clipperton Atoll; and type 3 was found only in Panama and the Galapagos Islands. Each type harboured a different *Symbiodinium* 'species lineage' in Clade C, and only type 1 associated with the 'stress-tolerant' *Symbiodinium glynni* (*D1*). The accurate delineation of species and implementation of a proper taxonomy may profoundly improve our assessment of *Pocillopora*'s reproductive biology, biogeographic distributions, and resilience to climate warming, information that must be considered when planning for the conservation of reef corals.

Keywords: biogeography, coral systematics, hybridization, *Pocillopora*, reciprocal monophyly, species boundaries, Tropical Eastern Pacific

Received 28 July 2010; revision received 11 October 2010; accepted 18 October 2010

Introduction

Modern approaches to ecology, evolution and systematics increasingly rely on the power of molecular genetic analyses. Acquisitions of genetic data have improved our understanding of many ecological interactions and evolutionary relationships and have frequently resulted in substantial changes in the systematics of organism lineages. Indeed, molecular genetic data have supported and challenged the foundations of numerous taxonomic hierarchies based originally on traditional morphological analyses (Hillis 1987; Doyle 1997; Sites & Marshall 2003; Will and Rubinoff 2004). Cnidarians in the order Scleractinia (i.e. stony corals) comprise an ecologically important group whose systematics requires major revision. The evolutionary relationships among and within morphologically defined families and genera do not always agree with nucleotide sequence phylogenies (Romano & Palumbi 1996; Fukami *et al.* 2004b; Fukami 2008; Forsman *et al.* 2009). For example, it is now evident that extant reef-building corals comprise two

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divergent clades, the 'robust' and 'complex' corals (Romano & Palumbi 1996; Romano & Cairns 2000; Budd *et al.* 2010). Masked by evolutionary convergence, family groupings that comprise these ancient clades contain representatives that are para- and polyphyletic (Fukami 2008). For example, the Atlantic faviids and mussiids are genetically more similar to each other than faviids and mussiids in the Pacific, indicating that Caribbean and Indo-Pacific coral communities comprise evolutionarily distinct assemblages (Fukami *et al.* 2004b). Clearly the revision of scleractinian systematics has important implications for deducing the ecology and evolution of these organisms.

Species delimitation within various coral genera faces similar challenges to those found at higher taxonomic ranks. Irradiance levels, wave exposure, sedimentation, nutrient availability and competition may significantly influence colony morphology, calice size and polyp shape (reviewed in Todd 2008). The influence of external environmental factors on interindividual variability can therefore impede the species resolution using morphometric analyses (Veron 1995; Todd 2008). To overcome these challenges, comprehensive studies utilizing a combination of morphological, ecological and genetic data help to resolve, substantiate and/or revise taxonomic affinities among closely related species (Sites & Marshall 2003; De Queiroz 2007). Given the importance of corals to reef ecosystems, the ability to properly describe and deduce ecological patterns and processes demands that they be accurately and precisely classified (Hey *et al.* 2003; Bickford *et al.* 2007).

Corals in the genus *Pocillopora* are common and widely distributed throughout the Indo-Pacific (Veron 2000). Approximately 16 species (Veron 2000, 2002) are classified based on morphology. Eight species are found in the far Tropical Eastern Pacific (TEP; Table S1, Supporting information, *P. capitata, P. damicornis, P. effusus, P. eyudoxi, P. inflata, P. meandrina, P. verrucosa/elegans* and *P. woodjonesi*) where they are especially dominant and vital to the productivity of shallow marine communities (Cortes 1997; Glynn & Ault 2000). Instead of brooding their larvae, TEP *Pocillopora* broadcast their gametes during spawning presumably representing an adaptive shift caused by the isolation and relatively inhospitable environmental conditions characteristic of the region (Glynn *et al.* 1991). Recently, the analysis of the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA tandem array suggested that introgressive hybridization was common among TEP *Pocillopora* (Combosch *et al.* 2008) and could explain the morphological intermediates found between various morphospecies. Hybridization is thought to be important in the evolution of corals and that hybrids are potentially better adapted to environmentally variable and extreme habitats (Willis *et al.* 2006). Still, published studies of coral hybridization are limited to a few exceptional genera suggesting that general conclusions about the importance of hybridization to scleractinian ecology and evolution require further substantiation.

Pocillopora colonies in the TEP are known to differ in their response to anomalous warm- and cold-water episodes created by El Niño Southern Oscillations (ENSO) and the reverse processes of these events (Glynn *et al.* 2001; Reyes-Bonilla *et al.* 2002; LaJeunesse *et al.* 2010). Colonies usually associate with one of two distantly related species of *Symbiodinium*, one in Clade C and the other in Clade D whose distributions appear random from colony to colony irrespective of morphospecies and external environmental factors (LaJeunesse *et al.* 2008). This variation in symbioses among colonies explains why one individual loses its endosymbiotic algae, or 'bleaches', while an adjacent colony appears normal (Glynn *et al.* 2001; LaJeunesse *et al.* 2010). Indeed, associations with *S. glynni* (*D1 sensu* LaJeunesse *et al.* 2008) may be an important determinant for why some colonies survive during periods of severe physiological stress (LaJeunesse *et al.* 2010). Therefore, the symbiosis ecology of TEP *Pocillopora* is presented as an example of how variation in partner combinations is important for determining how coral populations may respond to climate warming.

This study was initiated to investigate questions about hybridization and symbiosis specificity among TEP *Pocillopora* using the mitochondrial open reading frame region (ORF, Flot & Tillier 2007) and ITS2 sequences in combination with population genetic data (seven microsatellite markers). We combined these analyses with the genetic identification of the resident algal symbiont. The evidence gathered was consistent with a single parsimonious, albeit unanticipated, conclusion that may finally explain unusual geographic differences in reproductive biology among *Pocillopora* while raising additional questions regarding the genetic basis of morphological variability among individuals. This investigation offers a model with which to approach future systematic analyses of closely related Scleractinia.

Methods

Field collections for genetic analyses

Colonies of *Pocillopora* with different morphologies were collected at locations throughout the TEP, including Mexico (GoC, Gulf of California; BB, Banderas Bay; REV, Revillagigedo Islands; and OAX, Oaxaca), the Clipperton Atoll (CLP), Panama (PAN) and the Galapagos Islands (GAL) (Fig. 1). Colonies were identified in

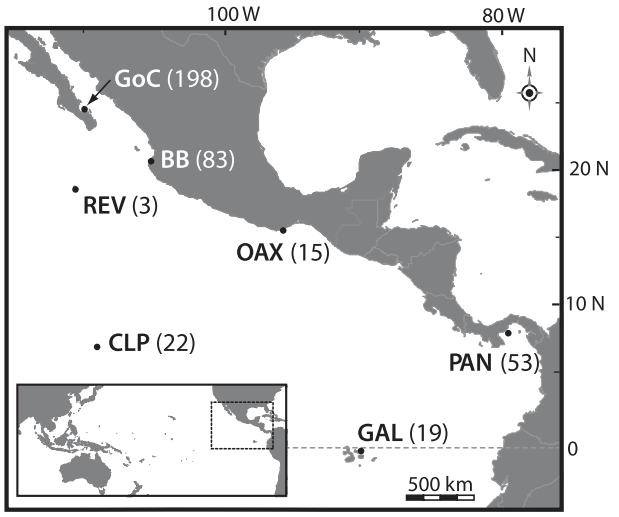

Fig. 1 Sampling locations for *Pocillopora* in the Tropical Eastern Pacific (GoC, Gulf of California; BB, Banderas Bay; OAX, Oaxaca; REV, Revillagigedo Islands; PAN, Gulf of Panama; CLP, Clipperton Atoll; GAL, Galapagos Islands). The numbers of samples analysed from each location are in parentheses.

the field by their general morphological characteristics including branch shape and thickness, size and uniformity of verrucae and overall colony morphology as described in the text and pictured in Veron (2000). Local experts verified these identifications in the field or from underwater digital photographs. Intermediate or variant morphologies that could not be assigned to a particular species morphotype were recorded as *Pocillopora* sp. Small fragments (<1 cm long) were acquired from the colonies using bone clippers. Fragments were fixed in ethanol or DMSO-NaCl preservation buffer (Seutin *et al.* 1991) and stored in the freezer at −20 °C.

DNA extractions

Genomic DNA extractions were performed on small skeletal fragments containing animal tissue using a non-toxic protocol modified from LaJeunesse *et al.* (2003). A small piece of skeleton and tissue was combined with glass beads (Ceroglass, Columbia, TN, USA) and 600 μl of a cell lysis solution (0.2 M Tris, 2 mM EDTA, 0.7% SDS, pH 7.6), and shaken at high speed using a BioSpec beadbeater. The extract was then incubated with proteinase K (20 mg/ml) at 65 °C for 1 h. After incubation, proteins were precipitated from solution using ammonium acetate (9 M) and the sample stored at −20 °C overnight. The frozen extract was centrifuged (10 000 ***g***), the supernatant removed and placed into a new tube, and the DNA precipitated from solution with 100% isopropanol and centrifuged (10 000 ***g*** for 5 min.). The DNA pellet was washed with 70% ETOH, air dried and resuspended in 75 μl of distilled water and stored at −20 °C.

PCR amplification and sequencing of animal DNA

The mitochondrial open reading frame (ORF) was amplified with the FATP6.1 and the RORF primers (Flot *et al.* 2008). For a subset of samples, the entire ITS2 as well as a portion of the 5.8S RNA gene of the nuclear ribosomal array were amplified using Scler5.8Sbforward and ITSrev primers (LaJeunesse & Pinzón 2007). Amplified products were sequenced with the forward primer using Big Dye 3.1 terminator mix (Applied Biosystems) following the manufacturers protocol on an ABI Hitachi 3730XL genetic analyzer.

DNA sequence chromatograms were reviewed and edited using Geneious Pro 5.0 (Drummond *et al.* 2009). Sequences were aligned by eye or using the software ClustalW with a gap-opening penalty of 15 and extension penalty of 6 (Thompson *et al.* 1994) and alignments exported into PAUP* (Swofford 2000). Separate unrooted phylogenies were constructed for the mitochondrial ORF and nuclear ITS2. Heuristic search and bootstrap (1000 replicates) were performed using maximum parsimony, neighbor joining and maximum likelihood methods. Additionally, a Bayesian analysis was run in Mr Bayes (Huelsenbeck & Ronquist 2001), using the HKG85 substitution model with a chain length of 1 100 000 and 100 000 burn-in.

Population genetic markers: microsatellite genotyping and data analysis

Allelic diversity and frequency were assessed using six published microsatellite loci: Pd3-002, Pd3-005, Pd2-006, Pd2-007, Pd3-008 and Pd3-009 (Starger *et al.* 2007) and a seventh new locus (Poc40). Previously published loci were optimized for the TEP samples by adding BSA (0.5 mg/ml). In three primer sets (Pd2-007, Pd3-008 and Pd3-009), the PCR buffer (NEB = 10 mM, Tris-HCl pH 8.3, 50 mM KCl, 25 mM $MgCl_2$) was replaced with the 10× PCR Buffer #3 in the SIGMA PCR optimization kit (Product #P2206; Buffer #3 = 100mM Tris-HCl pH 8.3, 250 mM KCl, 35 mM $MgCl_2$). Poc40 was found during the development of microsatellite markers for Clade D *Symbiodinium* (Pettay & LaJeunesse 2009). Amplification profiles for all loci followed the same conditions $[(94\ °C\ \ 2\ min)_1$—$(94\ °C\ \ 15\ s$—Ta $15\ s$—$72\ °C\ \ 30\ s)_{31}$—$(72\ °C$—$5\ min)_1]$ with the appropriate annealing temperature (Ta) (Table S2, Supporting information). Each microsatellite locus, with a labelled primer, was amplified in a separate PCR, then pairs of products from loci with different florescent labels and sizes were co-loaded on an ABI Hitachi

3730XL, using LIZ500 as a size standard. Allele sizes were scored in the software GeneMarker (Softgenetics, State College, PA, USA).

The frequencies of null alleles were estimated with two software packages; Microchecker (van Oosterhout *et al.* 2004) and INest (Chybicki & Burczyk 2008). INest includes an estimate of the selfing rates within populations in the calculation of null allele frequencies (Chybicki & Burczyk 2008). Additional tests of heterozygote deficiency were performed in Genepop on the Web (Raymond & Rousset 1995; Rousset 2008). The probability of identity (power of the markers to distinguish different genotypes) was calculated prior to detection and removal of matching multilocus genotypes (MLG) (GenAlex 6.3; Peakall & Smouse 2006). All further analyses were performed with one representative of each MLG characterized.

A Bayesian analysis using the software Structure V. 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003) was performed assuming admixture, correlated allele frequencies and specifying a location prior. Simulations included five iterations for each K value (K = 1–10), with 100 000 burn-in and 1 000 000 chain length. The most probable number of genetically homogeneous groups (K) was determined following the ΔK statistics procedure (Evanno *et al.* 2005) as implemented in Structure Harvester (Earl 2009). Graphics were generated with Distruct (Rosenberg 2004) after aligning all multiple runs for each K with Clumpp (Jakobsson & Rosenberg 2007). An additional Bayesian estimate of the number of clusters (K) was performed with the software Structurama (Huelsenbeck & Andolfatto 2007). Structurama assigns individuals to populations implementing the Gibbs variant of the Markov Chain Monte Carlo (MCMC) assuming a Dirichlet process. We ran this analysis with 1 000 000 MCMC chains treating the number of populations as a random variable (Scale = 1; Shape = 1).

Phylogenetic relations between MLG were constructed using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the program Neighbor from the PHYLIP package (Felsenstein 1995). The distance matrix was created using the Cavalli-Sforza and Edwards distance logarithm (Cavalli-Sforza & Edwards 1967), implemented in the software Microsat (Human Population Genetics Laboratory, Stanford University).

Principal coordinate analyses (PCoA) based on genetic distances implemented in GenAlex 6.3 (Peakall & Smouse 2006) were performed to detect patterns of association among genotypic data. Probability tests were performed to detect deviations from Hardy–Weinberg equilibrium (HWE) in the PCoA clusters using Genepop (Raymond & Rousset 1995; Rousset 2008). The significance of differentiation between the PCoA defined groups was assessed with an AMOVA, R_{st} and F_{st} calculations (GenAlex 6.3; Peakall & Smouse 2006).

The software NewHybrids was used to identify hybrid genotypes (Anderson & Thompson 2002). NewHybrids uses an MCMC procedure to distinguish individuals belonging to three categories: pure (Species 1 and Species 2), hybrids (i.e. F1 and F2) and backcrosses (i.e. Species 1—F1). Analyses were run without prior or any individual-specific assumptions, with 50 000 burin and 500 000 sweeps.

Molecular genetic identification of Symbiodinium

For identification of the resident symbiotic dinoflagellate in each sample, DGGE fingerprinting of the ITS2 region was employed to screen and sequence the numerically dominant intragenomic variant (for details and significance of this approach, see LaJeunesse 2002; LaJeunesse & Pinzón 2007; Thornhill *et al.* 2007). *Symbiodinium* specific primers (ITSintfor2 and ITS2clamp) were used to amplify the ITS2 region with a touchdown PCR protocol (LaJeunesse & Trench 2000). PCR products were analysed using Denature Gradient Gel Electrophoresis (DGGE) on an 8% acrylamide gel with 45–80% denature gradient (urea and formamide). Diagnostic bands in each distinctive ITS2-DGGE profile were characterized through excision, re-amplification and direct sequencing (LaJeunesse 2002; LaJeunesse *et al.* 2004a,b, 2008). The genetic diversity of the resident symbiont was further analysed using the noncoding sequence of the psbA minicircle following the protocol and using the primers 7.4-Forw and 7.8-Rev specified by Moore *et al.* (2003). Sequencing reactions were conducted using the forward primer (7.4-Forw). Sequences were aligned by eye and a phylogeny constructed based on maximum parsimony using PAUP* (Swofford 2000).

Results

Mitochondrial and nuclear markers defined three distinct lineages of Pocillopora *in the TEP*

The most phylogenetically informative characters of the mitochondrial ORF are in the first 600 bases (Flot & Tillier 2007); therefore, we sequenced using the forward primer and aligned the first 830 bases. Four unique haplotypes (HQ378758–HQ378761) were identified from 301 samples sequenced. Two of these sequences were similar and differed by two base pair substitutions (0.2%) while these differed from the other haplotypes by 14 and 18 base changes (1.7–2.7%), respectively. These haplotypes partitioned phylogenetically into three distinct groups with statistically supported branches (Fig. 2a).

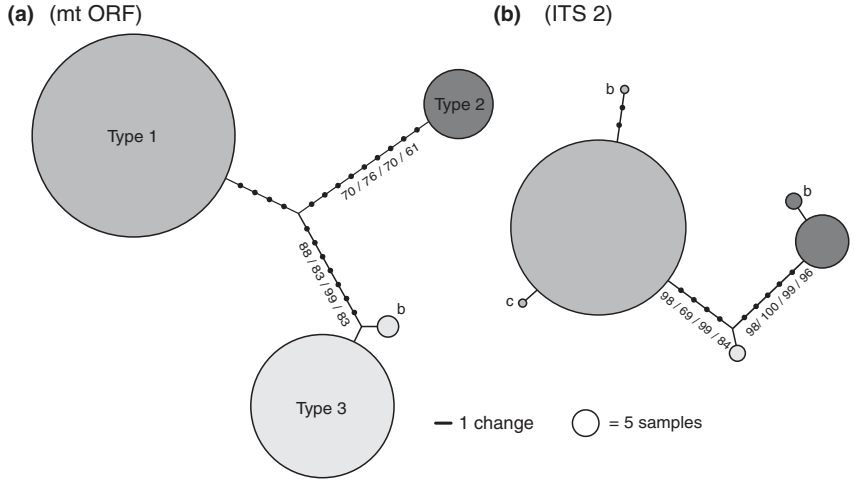

Fig. 2 Phylogenetic reconstructions of Tropical Eastern Pacific *Pocillopora* based on (a) the mitochondrial open reading frame (ORF; $n = 301$) and (b) the ITS2 region ($n = 50$). Each lineage was designated type 1 (medium grey), type 2 (dark grey) and type 3 (light grey). Topologies are drawn to the same scale and circle size is proportional to the number of samples possessing the same sequence (with the exception of type 1 ORF, $n = 257$). Numbers on the branches correspond to the support value of each branch after 1000 bootstrap replicates (Maximum likelihood/Neighbor Joining/Maximum Parsimony/Posterior probability MrBayes).

A total of six unique ITS2 haplotypes (HQ378752–HQ378757) ranging in size from 399 to 414 bases were recovered from direct sequencing of this spacer region from a total of 50 samples. Phylogenetic analyses of these sequences produced three well-supported lineages (>84%; Fig. 2b). These phylogenetic groupings matched those produced by the mitochondrial ORF. Samples that shared a particular ITS2 sequence also possessed the same corresponding mitochondrial sequence. Other than the existence of minor and rare sequence variants, no recombinant genotypes were found. Compared with mitochondrial sequence phylogenies, the *Pocillopora* diversity in the TEP comprises three genetically separate lineages designated as types 1, 2 and 3, respectively (Fig. 2a, b).

Microsatellite multilocus genotypes

The extent of genetic exchange among each phylogenetically defined type was examined with seven microsatellite loci. A total of 392 samples were analysed and from these 342 different MLG were identified ($G = 173$ in the GoC, 71 in BB, 2 in REV, 15 in OAX, 20 in CLP, 42 in PAN, 19 in GAL, abbreviations as in Fig. 1). The power of these markers to accurately distinguish between closely related genotypes and those produced by asexual reproduction was relatively high (probability of identity = 4.2×10^{-6}; Waits *et al.* 2001). The largest number of repeated MLGs were found in the GoC ($n = 8$), BB ($n = 5$) and PAN ($n = 8$) indicating the contribution of clonal propagation in population demographics of these corals. On two occasions, a MLG from the GoC was also found in BB.

Bayesian analysis performed in Structure and Structurama revealed three distinct populations, each corresponding to types 1, 2 and 3, respectively (Fig. 3a and Fig. S1b, Supporting information). Analyses using a greater cluster number (i.e. $K = 4$–10) did not further subdivide the data into separate populations beyond those already established. The largest population of related genotypes (type 1) comprised samples from all locations (n = GoC 173, BB 71, REV 2, OAX 15, CLP 9, PAN 22, GAL 16) and displayed significant deviations from HWE ($P < 0.05$) for all loci (Table S3, Supporting information). The other two clusters comprised genotypes that corresponded to type 3 (PAN, $n = 20$; GAL, $n = 3$) and genotypes that corresponded to type 2 (CLP, $n = 11$). HWE deviations were not significant in any locus from types 2 and 3. A second STRUCTURE analysis performed using the genotypic diversity of only type 1 colonies revealed limited geographic partitioning of populations across a latitudinal gradient in the TEP (Fig. S1, Supporting information). However, pairwise comparisons of F_{st} and R_{st} values, after Bonferroni corrections, showed no statistical significance among type 1 populations (Table 4a, b, Supporting information). Finally, analyses targeting sympatric populations of types 1 and 2 from CLP ($n = 22$) and types 1 and 3 from PAN ($n = 42$) showed the same strong genetic differentiation as indicated by the region wide analysis of Fig. 3a (Fig. S3a, b, Supporting information).

The Cavalli-Sforza genetic distance matrix between MLG used to generate a UPGMA phylogeny with Neighbor (Felsenstein 1995) revealed that these genotypes grouped similarly to the ORF and ITS2 phylogenies. The resulting tree topology formed three clusters

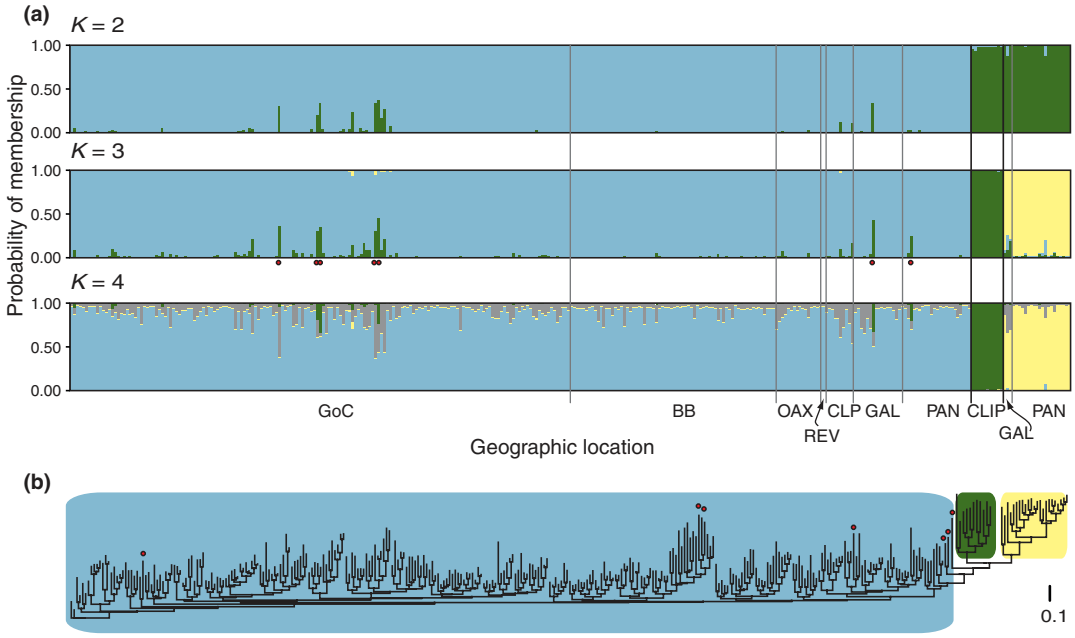

Fig. 3 Estimated population structure using Bayesian clustering (a) and Unweighted Pair Group Method with Arithmetic Mean phylogenetic reconstruction (b) of 342 multilocus genotypes (based on seven microsatellite loci) of Tropical Eastern Pacific *Pocillopora* spp. The separate populations identified correspond to the genetic groupings defined by ORF and ITS2 sequences. Dots mark seven putative hybrids of type 1 and type 3 identified by the software NewHybrids (abbreviations of geographic locations correspond to those in Fig. 1 and the colours blue, green and yellow coincide with type 1, 2 and 3, respectively). The plot figure shown for a given *K* is based on the composite probabilities of five independent statistical runs at that *K*.

matching the three lineages described by the mitochondrial and nuclear ribosomal DNA (Fig. 3b).

A PCoA analysis separated MLGs into three significant clusters, or groups (Fig. S2a, Supporting information). An AMOVA (R_{st} = 0.368; P = 0.01) revealed differences between the three groups with F_{st} pairwise values ranging from 0.360 to 0.454 all significant to the P = 0.01 level. These clusters match with those defined by Structure and by the ORF and ITS2 sequence data. That is, colonies with a particular ORF and ITS2 sequence type also shared closely related MLG.

Bayesian analysis of genotypes implemented in NewHybrids revealed the presence of seven individuals (2%; n = 342) with various levels of admixture that may be the product of recent hybridization events, all of them belonging to the *F*2 category (with *Q*-values between 0.1198 and 0.9928). Structure showed 6 of these individuals (dots in Fig. 3) as mixtures of type 1 and 2 and Structurama placed them close to type 2 (Fig. S2b, Supporting information) but the UPGMA distance-based tree consider these genotypes well within the type 1 lineage (Fig. 3b).

Symbiodinium *'species' associated with* Pocillopora

Internal transcribed spacer 2-DGGE analysis of all samples identified four distinct *Symbiodinium*. Of these, three belonged to Clade C (*C1b-c*, *C1d* and *C1ee*; Fig. 4a, b) and another was a member of Clade D (*S. glynni*), all except *Symbiodinium C1ee* were previously characterized from *Pocillopora* (LaJeunesse *et al.* 2004a,b, 2008). In most of the samples, the DGGE fingerprinting detected a single dominant species with mixtures of *C1b-c* and *S. glynni* detected in approximately 3% of colonies. *Symbiodinium C1b-c* and *S. glynni* were found in association with only type 1 while colonies characterized as type 3 harboured only *C1d*. Finally, type 2 colonies from the CLP (n = 11) harboured *C1ee* exclusively. Phylogenetic reconstructions using the first half of the noncoding region of psbA minicircle (~500–600 bases) differentiated these DGGE-ITS2 types into three well-supported lineages (Fig. 4b). Interindividual variability further separated these symbionts into distinct phylogeographic groupings. For example, sequences of *C1b-c* from PAN were more similar to each other than to those from the CLP (Fig. 4b; GenBank accession nos for *C1b-c*: HQ336237–HQ336255; *C1d*: HQ336231–HQ336236; and *C1ee* HQ336225–HQ336230).

Discussion

The identification of species diversity is particularly critical when investigating questions pertaining to

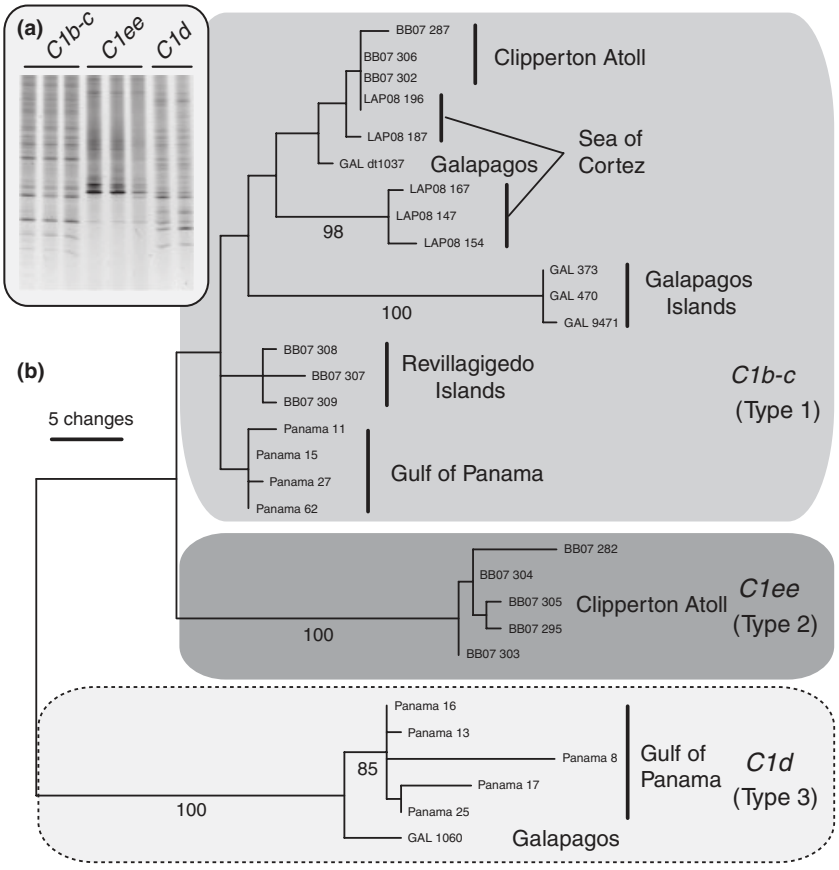

Fig. 4 (a) Representative DGGE ITS2 fingerprints of Clade C *Symbiodinium* found in symbiosis with Tropical Eastern Pacific *Pocillopora*. (b) Phylogenetic relationships between the three Clade C *Symbiodinium* (*C1b-c*, *C1ee* and *C1d*) based on the hyper-variable non-coding region of the psbA minicircle. The location origins of each sequence are provided showing distinct phylogeographic patterning. *Symbiodinium glynni* (*D1*) was found to associate with only type 1 colonies (not shown). Numbers below branches are bootstrap support values based on 1000 replicates.

physiology, ecology and evolution. Wrongly identified species, because of the presence of cryptic or overestimated diversity, has often contributed to false perceptions about biological patterns and processes (Bickford *et al.* 2007). Among scleractinians, for example, the apparent incongruence between genetic and morphological data has led to a questioning of traditionally diagnostic morphological traits in numerous cases, often indicating the need for significant changes in coral taxonomy and systematics (Romano & Palumbi 1996; Fukami *et al.* 2004b, 2008; Forsman *et al.* 2009; Budd *et al.* 2010). Presently there is considerable interest in the biology of reef-building corals especially in determining their capacity to adjust to climate warming (e.g. Hughes *et al.* 2003). The findings discussed below and contributions from recently published studies demonstrate that the taxonomic classifications of many coral lineages may need revision and that when instituted may improve our understanding of these important organisms. Indeed, the convergence of data based on phylogenetic patterns, population genetic makers and symbiosis ecology unequivocally partitions TEP *Pocillopora* into three natural groups that do not correspond to traditional taxonomic schemes.

The recognition that this genus comprises three distinct lineages (i.e. species) that relate little to morphology (Fig. 5a) indicates that the taxonomy of *Pocillopora* based on the morphospecies concept is flawed. These findings suggest (i) research into the underlying causes of morphological variation is needed, (ii) certain coral species may ultimately require molecular genetic analyses for identification and that (iii) a taxonomic revision of the genus *Pocillopora* based on the congruence of various genetic markers may establish their true diversity, improve understanding of ecological and geographic distributions and explain differences in reproductive biology (Glynn *et al.* 1991; Souter 2010).

High interindividual variation in colony morphology

Investigations of *Pocillopora* ecology in the TEP have relied on morphospecies classifications for decades

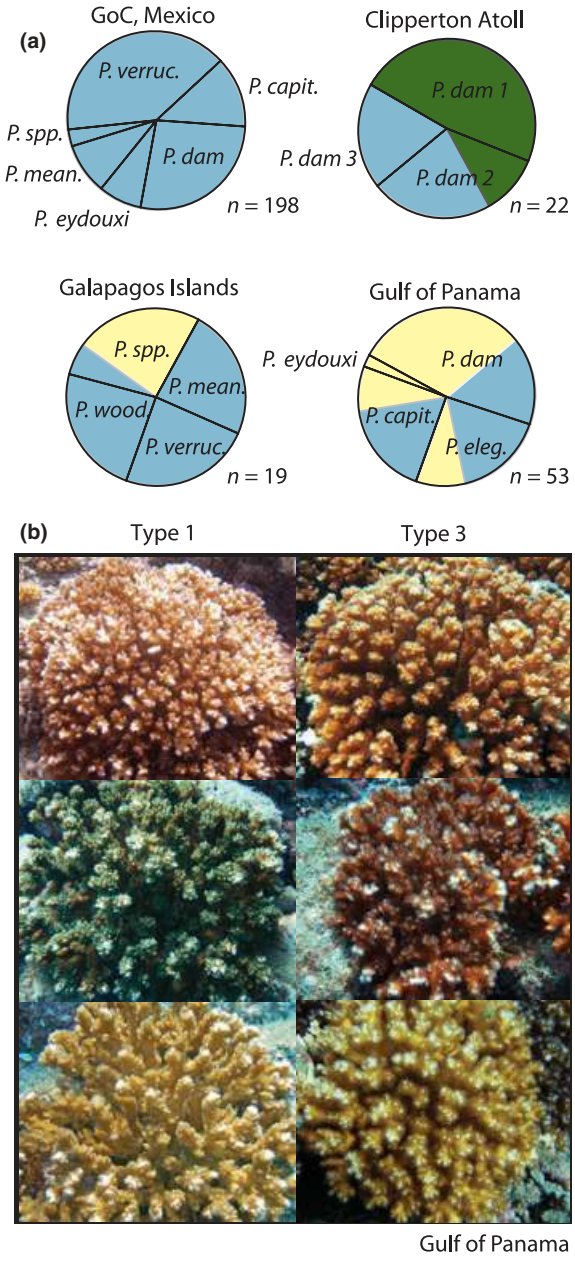

Fig. 5 Correspondence between colony morphology and genetic identity. (a) Pie charts representing the discordance between morphology and genetics among types 1 (blue), 2 (green) and 3 (yellow). (b) Morphological variability and similarity among colonies identified as types 1 and 3 in the Gulf of Panama, respectively.

(Cortes 1997; Glynn & Ault 2000; see references in Table S1, Supporting information). As stated in the introduction, formal descriptions of scleractinians are traditionally based on colony and skeleton morphology, yet phenotypic plasticity, interindividual variation and biogeographic variation sometimes confound accurate species identifications (Veron 1995). Tropical Eastern Pacific *Pocillopora* spp. appear to exhibit high levels of morphological variability in colony shape, branch size, as well as verrucae shape and density and substantial variation in colony morphology exists to the extent that one morphospecies grades to another among members of the same population and from the same environment (Veron & Pichon 1976).

The *Pocillopora* sampled in the GoC and mainland Mexico appear to embody a single lineage comprising *damicornis*-like, *meandrina*-like, *verrucosa*-like and *capitata*-like colony morphologies (Figs 5a and 6). This variation in morphology and existence of numerous intermediates between morphospecies, especially among colonies in the GoC, may relate to relaxed competition for resources (Grant 1972). This 'character release' has been proposed to explain the variability of the *Montastraea annularis* species complex based on fossil and living specimens from the Caribbean (Pandolfi *et al.* 2002) and/or hybridization (Fukami *et al.* 2004a).

In Panama, where types 1 and 2 coexist, there was little correspondence between colony morphology and genetics (Fig. 5b and Fig. S3b, Supporting information). Therefore, we were unable to assign traditional morphospecies binomials to these genetic groupings. *Pocillopora* type 2 may have greater morphological uniformity and actually correspond to *Pocillopora effusus* proposed originally to be endemic to the Clipperton Atoll (Fig. 5a; Glynn *et al.* 1996), although there are reports of this morphospecies in Mexico (Reyes-Bonilla 2003). Ultimate determination of the genetic basis of branch geometry, size and distribution of verrucae may eventually show that, in some *Pocillopora* populations, these traits are more akin to interindividual variants (allelic variation) than markers for species identity. The application of genomic approaches may provide information necessary to pursue these questions (Ball *et al.* 2002).

Congruence between phylogenetic and population genetic data

Members of lineages designated types 1, 2 and 3 possessed a characteristic ORF sequence and a corresponding ITS2 sequence (Fig. 2). The congruence, or reciprocal monophyly, of nuclear and mitochondrial sequences indicates that these lineages are evolutionarily divergent with little or no effective genetic recombination and/or hybrid introgression occurring between them (Avise & Wollengerg 1997). The analysis of population genetic data substantiates that types 1, 2 and 3 constitute reproductively isolated populations, or species (Fig. 3a, b and Fig. S1, Supporting information). Numerous alleles at various loci were unique to a particular type and/or allelic frequencies differed significantly between types (Table S3, Supporting

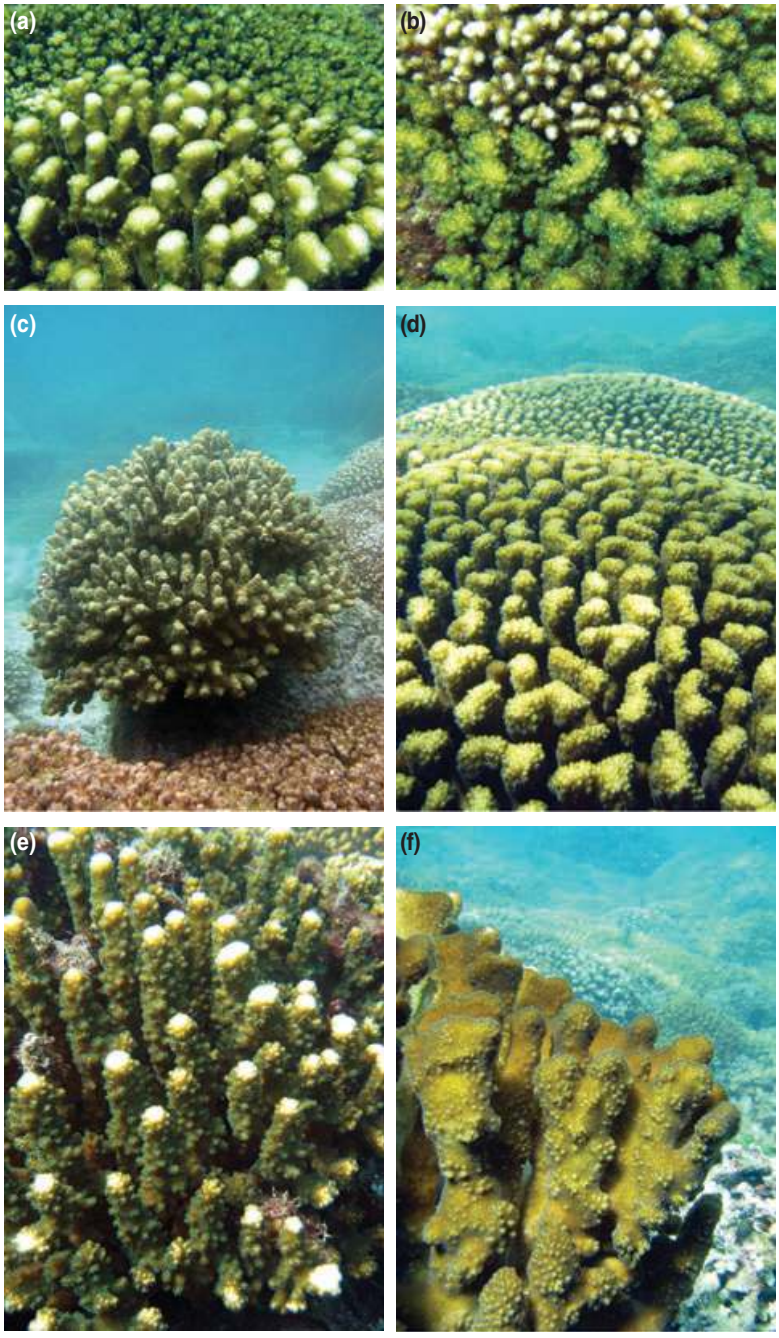

Fig. 6 Variation in morphology among type 1 colonies in the Gulf of California including morphospecies (a) *Pocillopora capitata*, (b) *P. damicornis* (above) and *P. verrucosa* (below), (c) *P. verrucosa*, (d) *P. meandrina*, (e) *P. capitata* and (f) *P. elegans*.

information). Colonies of type 1 throughout the TEP were far more similar to each other in allelic composition than to colonies of types 2 or 3 at locations where they exist sympatrically (GAL and PAN for type 1 and 3, and CLP for types 1 and 2; Fig. S3a, b, Supporting information). Indeed, there was no clear subdivision among type 1 populations throughout the EP indicating that gene flow and/or dispersal occurs at a high enough regularity across the region to homogenize allelic frequencies (Fig. 3a and Fig. S1, Supporting information).

In search of hybridization

It is presumed that hybridization is common among closely related coral lineages (Veron 1995; Willis *et al*. 2006) especially among synchronous broadcast spawning *Acropora* (van Oppen *et al*. 2000, 2001; Vollmer & Palumbi 2002). These conclusions, however, are based on a small number of reports involving few genera. Combosch *et al*. (2008) recently added to these studies and concluded that hybridization was common among morphospecies *P. damicornis*, *P. inflata*, *P. effusus*, *P. elegans* and

P. eydouxi from Panama and Clipperton based on ITS2 sequence variation and polyphyletic tree topologies. It should be noted that much of the sequence variability characterized by Combosch *et al.* (2008) was generated from cloning and sequencing PCR products of rDNA, a method shown to commonly recover low copy number intragenomic variants and pseudogenes (LaJeunesse & Pinzón 2007; Fig. S4 (Supporting information) is a comparison, based on maximum parsimony, of the ITS2 data reported in Fig. 2b with that reported by Combosch *et al.* 2008;. Note that types 1, 2 and 3 discussed in this paper do not correspond to their ITS2 clades I, II and III). While one or two sequences are usually most common among rDNA, significant intragenomic variability in rDNA often exists in eukaryote genomes. Either direct sequencing or employing techniques that target the numerically dominant sequence variant are recommended when analysing and comparing rDNA from closely related taxa (LaJeunesse & Pinzón 2007; Thornhill *et al.* 2007). Furthermore, it is probably inappropriate to use ITS sequences as population genetic markers because ribosomal genes exist in multiple copies and evolve differently and more slowly than single copy nuclear loci (Dover 1982).

Population genetic data presented here indicate that *Pocillopora* types 1, 2 and 3 rarely if at all hybridize (~2%; $n = 342$, see also Fig. 3b and Fig. S2b, Supporting information) and is in direct contrast to the conclusions of Combosch *et al.* (2008). If the different *Pocillopora* morphospecies examined by Combosch *et al.* (2008) were instead members of the same species, as is probably the case, then it is not surprising that their genetic data indicated significant evidence for recombination. Future assumptions of species identity may benefit by analysing population genetic data using a program like STRUCTURE (Pritchard *et al.* 2000) that distinguishes gene pools irrespective of morphological, geographic or taxonomic labels. Based on these results, an *a posteriori* assessment of species diversity and/or estimate of hybridization would follow.

Indeed, most genetic-based investigations of coral reproduction, ecology and evolution begin with the position that morphology is the *de facto* metric by which to identify species (e.g. Miller & Benzie 1997; van Oppen *et al.* 2001; Diekmann *et al.* 2003; Souter *et al.* 2009). This *a priori* assumption may wrongly influence interpretations of hybridization without considering the alternative possibility that more than one morphospecies constitutes a species, or that a single morphospecies comprises multiple cryptic taxa (Souter 2010). For example, allozyme studies involving several *Platygrya* morphospecies collected from the Great Barrier Reef (GBR) by Miller & Benzie (1997) found high genetic exchange and no relationship between genotype and morphospecies. In addition to these findings, it was observed that *Platygryra* on the GBR exhibit identical ecological distributions, all are sympatric, and fertilization success is similar within and between morphospecies (Miller & Babcock 1997). While these data point to one logical conclusion, the possibility that *Platygryra* morphospecies on the GBR may actually comprise a single species was never directly proposed and underscores how the sanctity of traditional taxonomic schemes restrains the interpretation of modern genetic data.

Heterogeneity in the reproductive strategies of pocilloporid corals including differences in gamete buoyancy, temporal separation in spawning times and differences in their mode of spawning (broadcast spawning vs. brooding) substantially reduces the probability of sexual recombination among species of *Pocillopora* (Kinzie III 1996). This contrasts with community assemblages of *Acropora* where numerous species synchronously mass spawn (Harrison & Wallace 1990) thus providing frequent opportunities for hybridization. Indeed, experimental crosses show that many *Acropora* morphospecies can hybridize (Willis *et al.* 1997) and genetic data indicate that such events occur with some frequency (van Oppen *et al.* 2000, 2001; Vollmer & Palumbi 2002). While hybridization may have contributed to the significant radiation of *Acropora* diversity since the Miocene-Pliocene boundary (Fukami *et al.* 2000; Hatta *et al.* 1999; van Oppen *et al.* 2001), a lack of hybridization may explain why diversity in the genus *Pocillopora* is significantly lower (Kinzie III 1996).

These findings may resolve speculation about why '*P. damicornis*,' a species that typically broods its larvae, undergoes broadcast spawning in the TEP (Glynn *et al.* 1991). Based on ORF sequence comparisons, *'P. damicornis'* in Hawaii is genetically different than the *'P. damicornis'* identified in the TEP (Flot *et al.* 2008; data not shown), a study of the reproductive biology of types 1, 2 and 3 may identify different strategies that correspond instead to these lineages rather than local adaptation to biotic and abiotic factors as previously proposed (Stimspon 1978; Richmond 1985). Indeed, *Pocillopora meandrina* in Hawaii appears to be a broadcast spawner (Stimspon 1978) and is a member of the type 1 group (Flot *et al.* 2008, unpublished data).

Symbiont specificity provides insight into coral species identity

One of the most important ecological interactions in the lives of a reef corals is their association with dinoflagellates in the genus *Symbiodinium*. Based on the combined analyses of ITS2-DGGE fingerprinting and sequencing of the noncoding region of the psbA minicircle (Fig. 4b), four 'species lineages' of symbiont, three in

Clade C and one in Clade D were identified from the samples examined during this study. Most importantly, each of these symbionts associated with only one type of *Pocillopora* (Fig. 4). Host cell environments (i.e. host-habitat) and interspecific competition probably exert strong selective pressures that contribute to ecological specialization and subsequent speciation among symbionts (Moulder 1979; Coffroth *et al.* 2001; LaJeunesse 2005). It might be that genetic differences between types 1, 2 and 3 influence the molecular/cellular interactions, contributing to the observed differences in host–symbiont specificity (LaJeunesse *et al.* 2004b, 2008; Rodriguez-Lanetty *et al.* 2006). Among the Clade C *Symbiodinium* identified in the TEP, *Symbiodinium C1d* occurs also in '*Pocillopora damicornis*' from Hawaii (LaJeunesse *et al.* 2004b). *Symbiodinium C1b-c* is probably a geographic variant of *C1c* associated with western and central Pacific *Pocillopora* spp. (central and southern GBR, LaJeunesse *et al.* 2003, 2004a; Sampayo *et al.* 2007; and found in *P. meandrina/eydouxi* from Hawaii, LaJeunesse *et al.* 2004b). *Symbiodinium C1ee* harboured by type 2 colonies is unique and, together with its host, may be endemic to the TEP.

The high fidelity exhibited by these symbioses may limit how certain partnerships respond to climate warming. Only type 1 associated with *Symbiodinium glynni* (found in approximately 230 of 290 colonies). Colonies with this symbiont are more resistant to dissociation, or 'coral bleaching', when exposed to environmental stressors (Glynn *et al.* 2001; LaJeunesse *et al.* 2007, 2010). This physiological resistance to thermal stress and apparent tolerance to episodes of turbidity may in part explain the ecological dominance of type 1 *Pocillopora* across the TEP (LaJeunesse *et al.* 2010). If the frequency and intensity of warm or cold-water events in the TEP increases, colonies of types 2 and 3 would probably be among the first to die out.

The biogeography of Pocillopora *types in the TEP and evidence of broader Indo-Pacific distributions*

Distinctive geographic provinces in the TEP are defined by similarity in community assemblages (Briggs 1974). The TEP partitions into three sub-provinces, the northern EP (NEP; GoC, central and southern Mexico and the REV), the equatorial EP (EEP; from Ecuador and Galapagos to Costa Rica) and the island EP (IEP; CLP and Malpelo Island) (Glynn & Ault 2000). Based on the scope of sampling in this study, the geographic distributions of types 1, 2 and 3 appear to correspond with these sub-provinces. In the EEP, *Pocillopora* types 1 and 3 were common inhabitants. At the CLP (part of the IEP), types 1 and 2 were both abundant, while type 1 occurred alone in the NEP (Fig. 7). Additional analysis

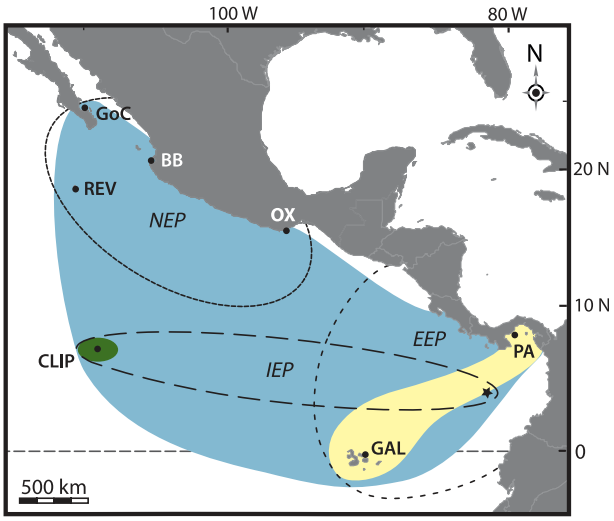

Fig. 7 Tentative geographic distribution of types 1 (blue), 2 (green) and 3 (yellow) in relation to biogeographic sub-provinces defined by Glynn & Ault (2000) (NEP, Northern Province; EEP, Equatorial province and IEP, Island Province). The star indicates the location of Malpelo Island in the IEP.

of samples from other coastal-shelf and island regions encompassed by Costa Rica, Colombia and mainland Ecuador is needed for the substantiation of these distributions. By determining the genetic identity of the morpho-species *P. capitata* and *P. eydouxi* that dominate communities at Malpelo Island (Garzón-Ferreira & Pinzón 1999), comparisons with the diversity found at the CLP would test whether IEP is a distinct biogeographic province.

Comparisons with published data identify types 1 and 3 in other Indo-Pacific regions. Using the mitochondrial ORF sequence as a tentative proxy for 'species' identification, type 1 exists in Hawaii and corresponds to the morphospecies of *Pocillopora meandrina/eydouxi* while type 3 also occurs in Hawaii, but corresponds to the morphospecies of *P. molokiensis* (Flot *et al.* 2008). The ORF of type 3 also matches with sequences of the 'NF-type' of '*P. damicornis*' described by Souter (2010) in the western Indian Ocean. Based on these preliminary data, types 1 and 3 are widely distributed suggesting that further sampling throughout the Indo-Pacific is needed to determine the extent of their geographic range and degree of genetic connectivity.

Multifaceted approach in delimiting species of Scleractinia

The application of genetic analyses in the systematics of corals is steadily increasing and many have identified inconsistencies between morphology, gene sequence similarity and ecology (Marquez *et al.* 2002; Diekmann

et al. 2003; Fukami *et al.* 2004a; Flot *et al.* 2008; Stefani *et al.* 2007; Bongaerts *et al.* 2010; Souter 2010). The recent analysis of '*Pocillopora damicornis*' from the western Indian Ocean concluded that this morphospecies actually comprises two distinct species (Souter 2010). In contrast, Hawaiian morphospecies of *Pocillopora* are accurately differentiated by distinct mitochondrial ORF sequences with the minor exception that *P. meandrina* and *P. eydouxi* appear to comprise the same haplotype lineage (Flot *et al.* 2008). Furthermore, each lineage harbours a distinct symbiont (LaJeunesse *et al.* 2004b). While further verification is required, the *Pocillopora* populations in Hawaii appear to breed true to their morphology. Collectively, these data further indicate that other *Pocillopora* types requiring additional genetic classification exist. Furthermore, each type may exhibit distinctive morphological appearances that relate to different geographic regions, thus demanding the need for a comprehensive Indo-Pacific wide study of *Pocillopora* examining the congruence between morphology, phylogenetic similarity, population genetics and symbiosis ecology.

As demonstrated here, the challenges of understanding species boundaries and/or the importance of hybridization among closely related corals may be overcome by using population genetic markers to examine genetic exchange and/or symbiont specificity when phylogenetic patterns and morphology are in conflict. Based on our current data, the actual diversity of *Pocillopora* in the TEP appears to be substantially lower than indicted by morphology. The possibility that the CLP contains a rare coral species should significantly influence decisions about regional conservation. The propensity of type 1 to associate with *Symbiodinium glynni* (*D1*) may explain its dominance in the TEP, whereas populations of type 2 and 3 colonies, apparently unable to associate with *S. glynni*, are probably more susceptible to climate warming (Glynn *et al.* 2001; LaJeunesse *et al.* 2010).

Acknowledgements

The authors acknowledge the help of several collaborators including: H. Reyes-Bonilla, A. L. Cupul-Magaña and A. López-Pérez for sample collections and logistics in Mexico. P. Medina-Rosas and Kristie Kaiser provided samples from the Clipperton Atoll and Revillagegado Islands. M. Torchin and C. Schloeder (Smithsonian Tropical Research Institute-Panama) facilitated collections in Panama (permit number SC/A-1-09). I. Baums and A. Baker provided samples from the Galapagos Islands. Population genetic data were analyzed using the Computational Biology Service Unit at Cornell University (http://cbsuapps.tc.cornell.edu/) I. Baums, Z. H. Forsman, T. Hopkins, A. Stephenson, D. Geiser and D. Pettay provided constructive evaluations on early versions of this manuscript. S. Palumbi and two anonymous reviewers provided valuable comments and suggestions during the review process. This research was funded in part by Florida International University, The Pennsylvania State University, the National Science Foundation (IOB 544854) and an Alfred P. Sloan Scholarship to JP.

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JHP is interested in how reproductive boundaries arise in the speciation of scleractinian corals. TCL studies the ecological and co-evolutionary processes important in animal–microbe associations as well as the response of coral–algal partnerships to major changes in climate.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Morpho-species diversity in the genus *Pocillopora* from the Eastern Pacific, as reported by regional diversity inventories and coral atlases

Table S2 Characteristics and amplification conditions of the microsatellite loci used to analyse the population structure of eastern Pacific *Pocillopora* spp. (*n*, number of alleles; H_e, expected; and H_o, observed heterozygosity; F, frequency; and Ta, annealing temperature)

Table S3 Allelic frequencies per locus for *Pocillopora* types I, II and III and across types (Overall). Samples size, number of unique genotypes and number private alleles (bold) are shown by type and overall

Table S4 (a) Pairwise Population Fst Values (below diagonal) and corresponding probability values (above diagonal). Tests of significance with ($\alpha = 0.5$) are in bold-faced numerals. No significance in population differentiation was detected following Bonferroni corrections. (b) Pairwise Population Rst Values (below diagonal) and corresponding probability values (above diagonal). Tests of significance with ($\alpha = 0.5$) are in bold-faced numerals. No significance in population differentiation was detected following Bonferroni corrections

Fig. S1 Estimated population structure of type 1 colonies sampled throughout the far Tropical Eastern Pacific depicting limited population subdivision over a latitudinal gradient. The plot figure shown for a given *K* is based on the composite probabilities of five independent statistical runs at that *K*. Groupings with the high statistical support (black arrowhead) $K = 2$ appear to distinguishes 'high latitude' and 'low latitude' populations of type 1.

Fig. S2 (a) Principal coordinate analysis (PCoA) and (b) Structurama analyses of the multilocus genotypes ($n = 342$) obtained from samples collected in the EP. Colours correspond to three genetic groupings (blue, type 1; green, type 2; yellow, type 3). The branches of three putative 'F-2 hybrids' are in black.

Fig. S3 Genetic structure of *Pocillopora* populations containing (a) types 1 and 2 from the Clipperton Atoll and (b) types 1 and 3 from the Gulf of Panama. Despite being sympatric, each population exhibited strong genetic differentiation corresponding to the mitochondrial open reading frame region and nuclear ITS2 lineages present. There was no evidence of mixed genotypes to indicate hybridization.

Fig. S4 Phylogenetic similarity based on maximum parsimony of ITS2 sequences originating from bacterial cloning of PCR amplifications published by Combosch *et al.* (2008) in their analysis of Tropical Eastern Pacific *Pocillopora* with sequences generated by direct sequencing reported by this study (see Fig. 2b). The sequences corresponding to ITS2 clade III (Combosch *et al.* 2008) are encompassed within the dashed box.

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Table S1. Morpho-species diversity in the genus *Pocillopora* from the Eastern Pacific, as reported by regional diversity inventories and coral atlases.

	Mexico [a, b]	El Salvador [c]	Costa Rica [d, e]	Panama [f]	Colombia [g, h, i]	Galapagos [j, k, l]	Clipperton [m]
P. damicornis	X	X	X	X	X	X	X
P. elegans	X	X	X	X	X	X	
P. meandrina	X	X	X	X		X	
P. eydouxi	X		X	X	X	X	
P. inflata *	X		X	X		X	
P. capitata	X	X			X	X	
P. effuses *	X	X					X
P. verrucosa	X						
P. woodjonesi	X					X	
P. danae					X		
Total	9	5	5	5	5	7	2

[a]Reyes-Bonilla H (2003) Coral reefs of the Pacific coast of Mexico. In: *Latin American Coral Reefs* (ed. Cortes J), pp. 331-350. Elsevier, Amsterdam.

[b]Reyes-Bonilla H, Calderon LE, Cruz G*, et al.* (2005) *Atlas de corales petreos (Anthozoa:Scleractinia) del Pacifico mexicano* Centro de Investigacion cientifica y educacion superior de Ensenada, Mexico.

[c]Reyes-Bonilla H, Barraza JE (2003) Corals and associated marine communities from El Salvador. In: *Latin American Coral Reefs* (ed. Cortes J), pp. 351-360. Elsevier, Amsterdam.

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[k]Glynn P (2003) Coral communities and coral reefs of Ecuador. In: *Latin American Coral Reefs* (ed. Cortes J), pp. 449-472. Elsevier, Amsterdam.
[l]Glynn PW, Veron J, Wellington G (1996) Clipperton Atoll (eastern Pacific): oceanography, geomorphology, reef-building coral ecology and biogeography. *Coral Reefs* **15**, 71-99.
[m]Glynn PW (1999) *Pocillopora inflata*, a new species of Scleractinian coral (Cnidaria:Anthozoa) from the Eastern Pacific. *Pacific Science* **53**, 168-180.

Table S2. Characteristics and amplification conditions of the microsatellite loci used to analyze the population structure of eastern Pacific *Pocillopora* spp. (N = Number of alleles, H_e = expected and H_o = observed heterozygosity, F = frequency and Ta = annealing temperature)

Locus	Primer sequences	Motif	Dye	N	H_o	H_e	F	Range	Buffer	Ta*
Pd3-002[a]	ATCCGAATACAAGCGAAACG	AAC	HEX	10	0.459	0.646	0.289	180-207	NEB	55
	CAAAGCTTCTATCAGAAAATGCAA									
Pd3-005[a]	AGAGTGTGGACAGCGAGGAT	TGA	FAM	16	0.348	0.535	0.35	191-257	NEB	55
	GTTCCTTCGCCTTCGATTTT									
Pd2-006[a]	ATCTCCATGTGATCGGCATT	CA	FAM	12	0.503	0.727	0.308	186-208	NEB	55
	GTTCCCCCAGCTGAGAAGTT									
Pd2-007[a]	AAGAAGGTGTGGTATTTCAGAGGG	AC	FAM	17	0.646	0.867	0.255	236-468	Sig 3	60
	GGTGGATAAAGTATTTCTCACTCTTGG									
Pd3-008[a]	AGTTGAGGTTGTTGAAACATG	CTG	HEX	5	0.278	0.455	0.39	154-166	Sig 3	55
	TCCATGCAGAACCCC									
Pd3-009[a]	CCAATGCGTCCGTAGCTCTC	(CAA)	FAM	8	0.164	0.415	0.606	327-348	Sig 3	52
	ATCACCTAAAAATTTCAGTCCCTTACC	(GAG)								
Poc40[b]	GTTATTATATGGGTGTATGC	CAA	HEX	11	0.345	0.545	0.367	289-316	NEB	55
	CTCAAAGTGCGATTAAAGCC									

[a]Starger et al 2007 [b]This project. Sig 3 = 10X PCR Sigma Buffer #3, Product #P2206, 100mM Tris-HCl pH 8.3, 250mM KCl, 35 mM $MgCl_2$
NEB = 10mM, Tris-HCl pH 8.3, 50mM KCl, 25mM $MgCl_2$ * PCR Protocol (94°C 2 min)$_1$ (94°C 15sec – Ta 15sec – 72°C 30sec)$_{31}$ (72°C 5min)$_1$

Table S3. Allelic frequencies per locus for *Pocillopora* types I, II, and III and across types (Overall). Samples size, number of unique genotypes and number private alleles (**bold**) are shown by type and overall.

Locus	Allele	type I	type II	type III	Overall
Pd3-002	**180**	**0.002**	0.000	0.000	0.001
	183	**0.015**	0.000	0.000	0.013
	186	0.036	0.000	0.065	0.037
	189	0.063	0.409	0.391	0.096
	192	0.232	0.045	0.391	0.237
	195	0.565	0.455	0.130	0.532
	198	0.008	0.000	0.022	0.009
	201	**0.003**	0.000	0.000	0.003
	204	0.075	0.091	0.000	0.070
	207	**0.002**	0.000	0.000	0.001
Pd3-005	191	0.005	0.000	0.022	0.006
	194	0.065	0.000	0.022	0.060
	196	**0.036**	0.000	0.000	0.032
	200	0.023	0.500	0.000	0.037
	203	0.740	0.000	0.022	0.668
	206	0.058	0.091	0.022	0.057
	209	0.015	0.182	0.043	0.022
	212	0.042	0.000	0.870	0.096
	215	**0.002**	0.000	0.000	0.001
	218	0.003	0.227	0.000	0.010
	221	**0.002**	0.000	0.000	0.001
	224	**0.003**	0.000	0.000	0.003
	227	**0.002**	0.000	0.000	0.001
	233	**0.002**	0.000	0.000	0.001
	235	**0.002**	0.000	0.000	0.001
	257	**0.002**	0.000	0.000	0.001
Pd2-006	**186**	0.000	0.000	**0.043**	0.003
	188	0.002	0.000	0.043	0.004
	190	0.261	0.000	0.022	0.237
	192	0.360	0.000	0.130	0.333
	194	0.302	0.000	0.739	0.322
	196	0.054	0.136	0.022	0.054
	198	0.002	0.136	0.000	0.006
	200	0.005	0.091	0.000	0.007
	202	0.000	**0.273**	0.000	0.009
	204	0.010	0.045	0.000	0.010
	206	0.003	0.182	0.000	0.009
	208	0.002	0.136	0.000	0.006
Pd2-007	236	0.045	0.682	0.022	0.064
	260	0.104	0.318	0.000	0.104
	262	**0.002**	0.000	0.000	0.001
	264	**0.002**	0.000	0.000	0.001
	280	**0.029**	0.000	0.000	0.026
	282	0.089	0.000	0.022	0.082
	305	**0.093**	0.000	0.000	0.083

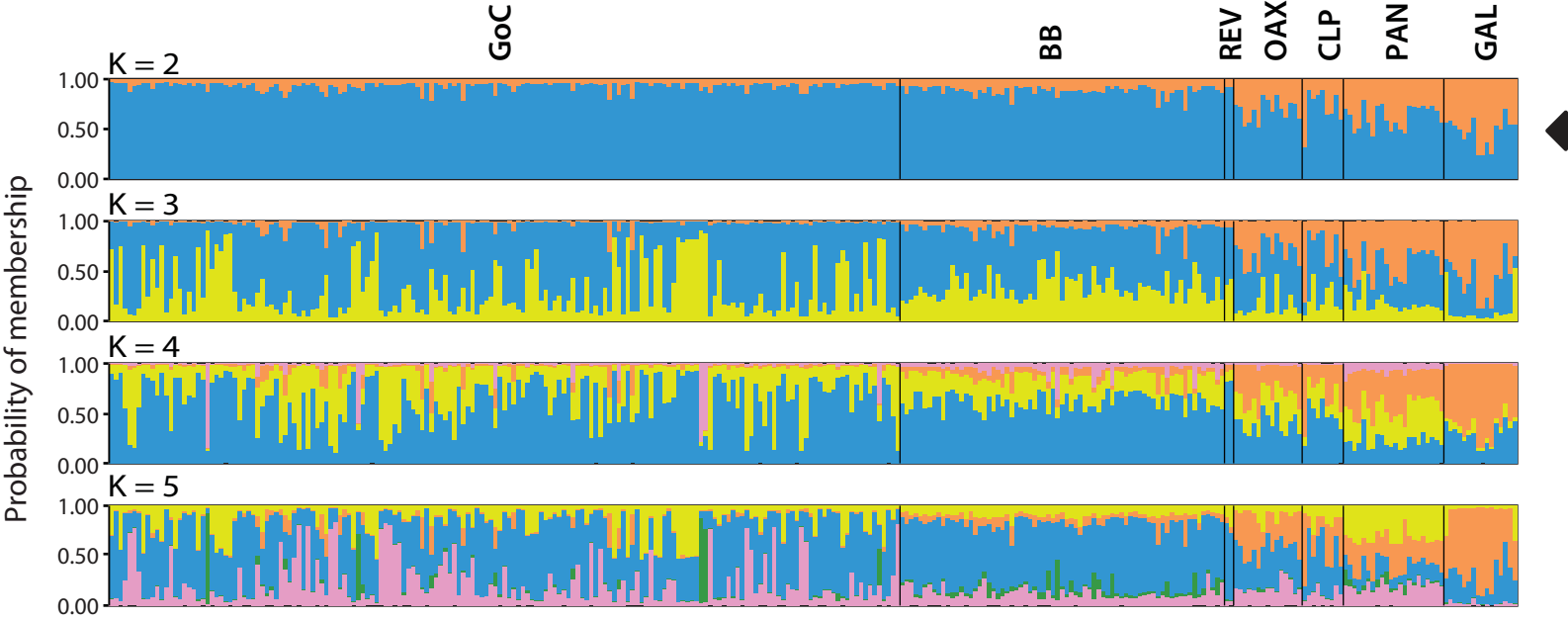
	329	0.024	0.000	0.152	0.032
	331	0.000	0.000	**0.783**	0.053
	353	0.281	0.000	0.022	0.254
	355	**0.005**	0.000	0.000	0.004
	371	**0.013**	0.000	0.000	0.012
	375	**0.162**	0.000	0.000	0.146
	398	**0.133**	0.000	0.000	0.120
	417	**0.002**	0.000	0.000	0.001
	464	**0.008**	0.000	0.000	0.007
	468	**0.008**	0.000	0.000	0.007
Pd3-008	**154**	**0.002**	0.000	0.000	0.001
	157	**0.062**	0.000	0.000	0.056
	160	0.781	0.273	0.000	0.712
	163	0.107	0.682	0.935	0.181
	166	0.049	0.045	0.065	0.050
Pd3-009	**327**	0.000	**0.091**	0.000	0.003
	331	0.010	0.045	0.022	0.012
	334	**0.075**	0.000	0.000	0.067
	337	0.016	0.545	0.891	0.092
	340	0.825	0.182	0.087	0.754
	342	0.013	0.136	0.000	0.016
	345	**0.060**	0.000	0.000	0.054
	348	**0.002**	0.000	0.000	0.001
Poc40	**289**	**0.003**	0.000	0.000	0.003
	292	**0.731**	0.000	0.000	0.658
	295	0.034	0.091	0.043	0.037
	298	0.008	0.000	0.522	0.042
	301	0.019	0.727	0.435	0.070
	304	**0.104**	0.000	0.000	0.094
	307	0.078	0.182	0.000	0.076
	310	**0.015**	0.000	0.000	0.013
	313	**0.006**	0.000	0.000	0.006
	316	**0.002**	0.000	0.000	0.001
Sample size		351	12	29	392
Unique Genotypes		308	11	23	342
Private Alleles		35	2	2	

Supplemental Table 4a. Pairwise Population Fst Values (below diagonal) and corresponding probability values (above diagonal). Tests of significance with (alpha = 0.5) are in bold-faced numerals. No significance in population differentiation was detected following bonforroni corrections.

	GoC	BB	REV	OAX	CLP	PAN	GAL
GoC	–	**0.010**	**0.020**	**0.020**	**0.030**	**0.010**	0.010
BB	0.008	–	**0.010**	**0.010**	0.070	**0.010**	0.010
REV	0.121	0.117	–	0.080	0.070	**0.030**	0.010
OAX	0.020	0.032	0.057	–	**0.030**	**0.010**	0.010
CLP	0.025	0.021	0.079	0.036	–	**0.010**	0.010
PAN	0.081	0.075	0.113	0.039	0.050	–	0.010
GAL	0.083	0.089	0.176	0.041	0.058	0.083	–

Supplemental Table 4b. Pairwise Population Rst Values (below diagonal) and corresponding probability values (above diagonal). Tests of significance with (alpha = 0.5) are in bold-faced numerals. No significance in population differentiation was detected following bonforroni corrections.

	GoC	BB	REV	OAX	CLP	PAN	GAL
GoC	–	0.070	0.140	0.350	**0.030**	0.310	**0.010**
BB	0.010	–	0.350	0.060	0.150	0.170	**0.010**
REV	0.089	0.000	–	0.130	0.380	0.100	**0.030**
OAX	0.000	0.032	0.192	–	**0.020**	0.370	0.120
CLP	0.113	0.030	0.000	0.205	–	0.080	**0.020**
PAN	0.000	0.010	0.114	0.000	0.126	–	0.060
GAL	0.149	0.213	0.340	0.054	0.351	0.091	–


GoC
BB
REV
OAX
CLP
PAN
GAL
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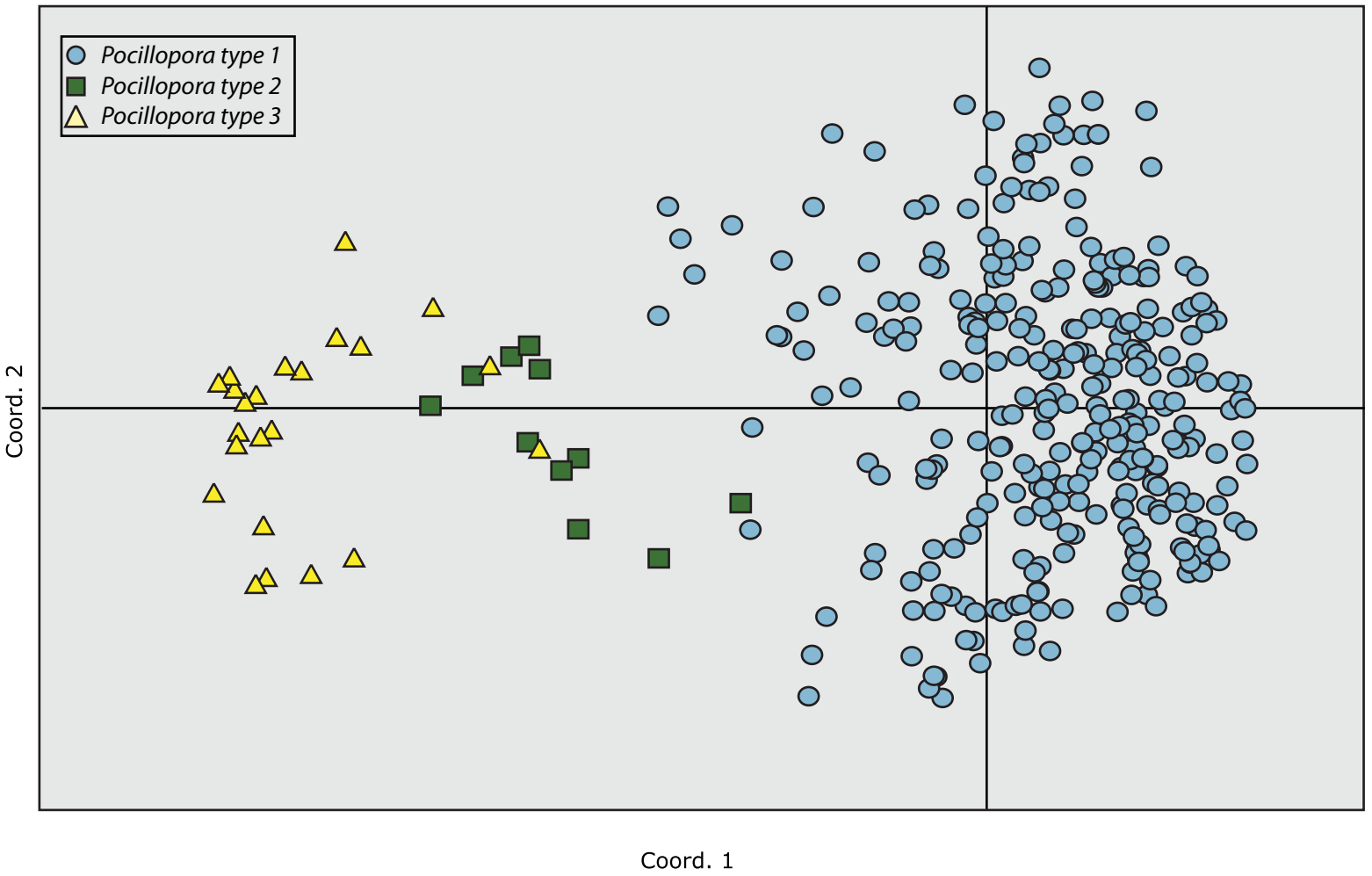
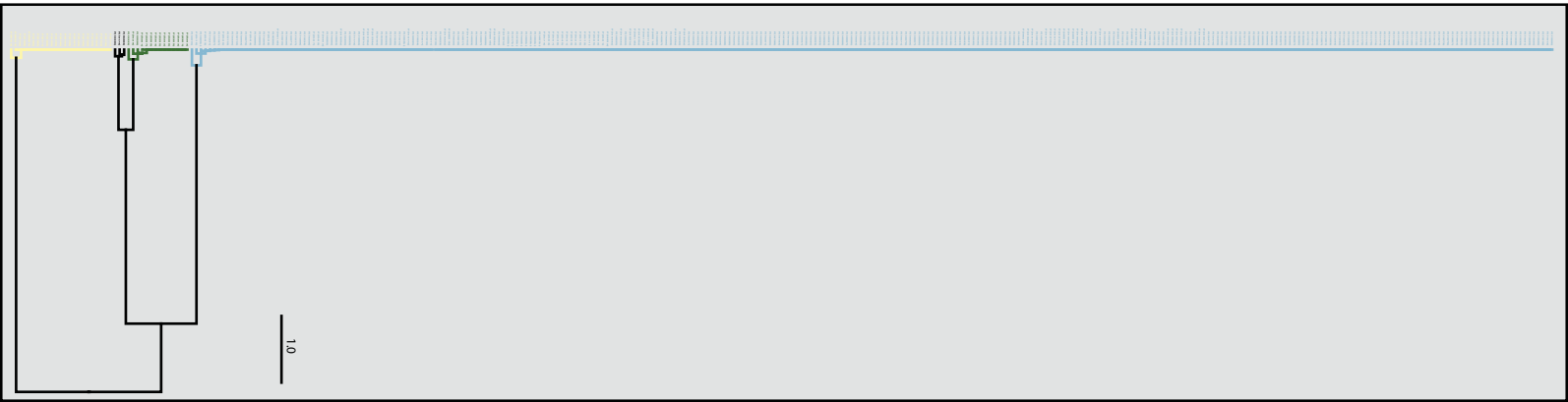
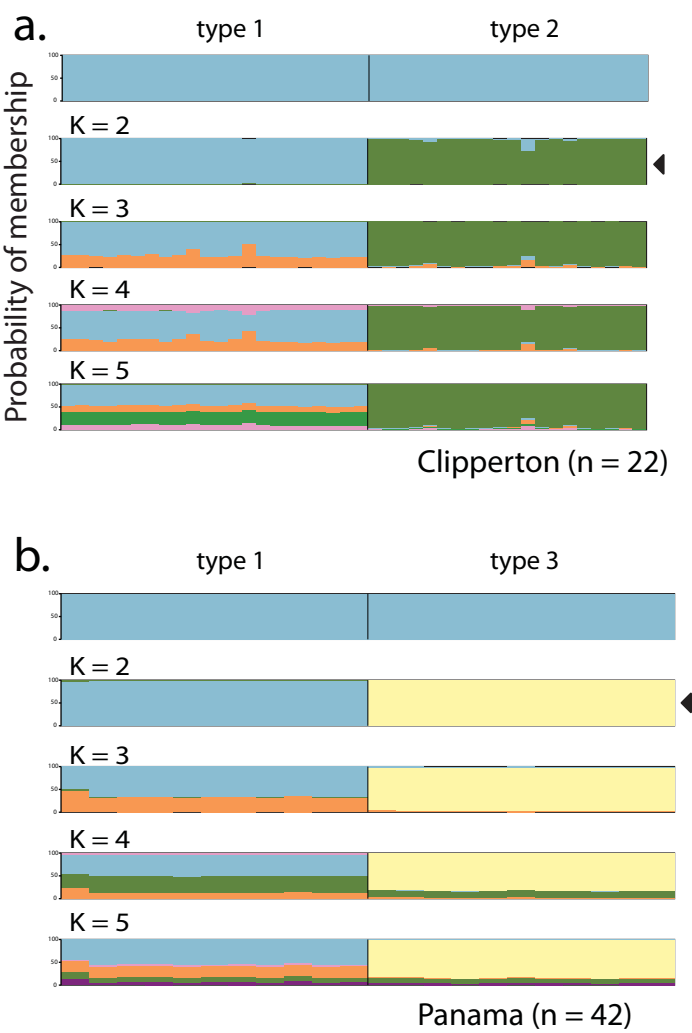
0.50
0.00
Probability of membership

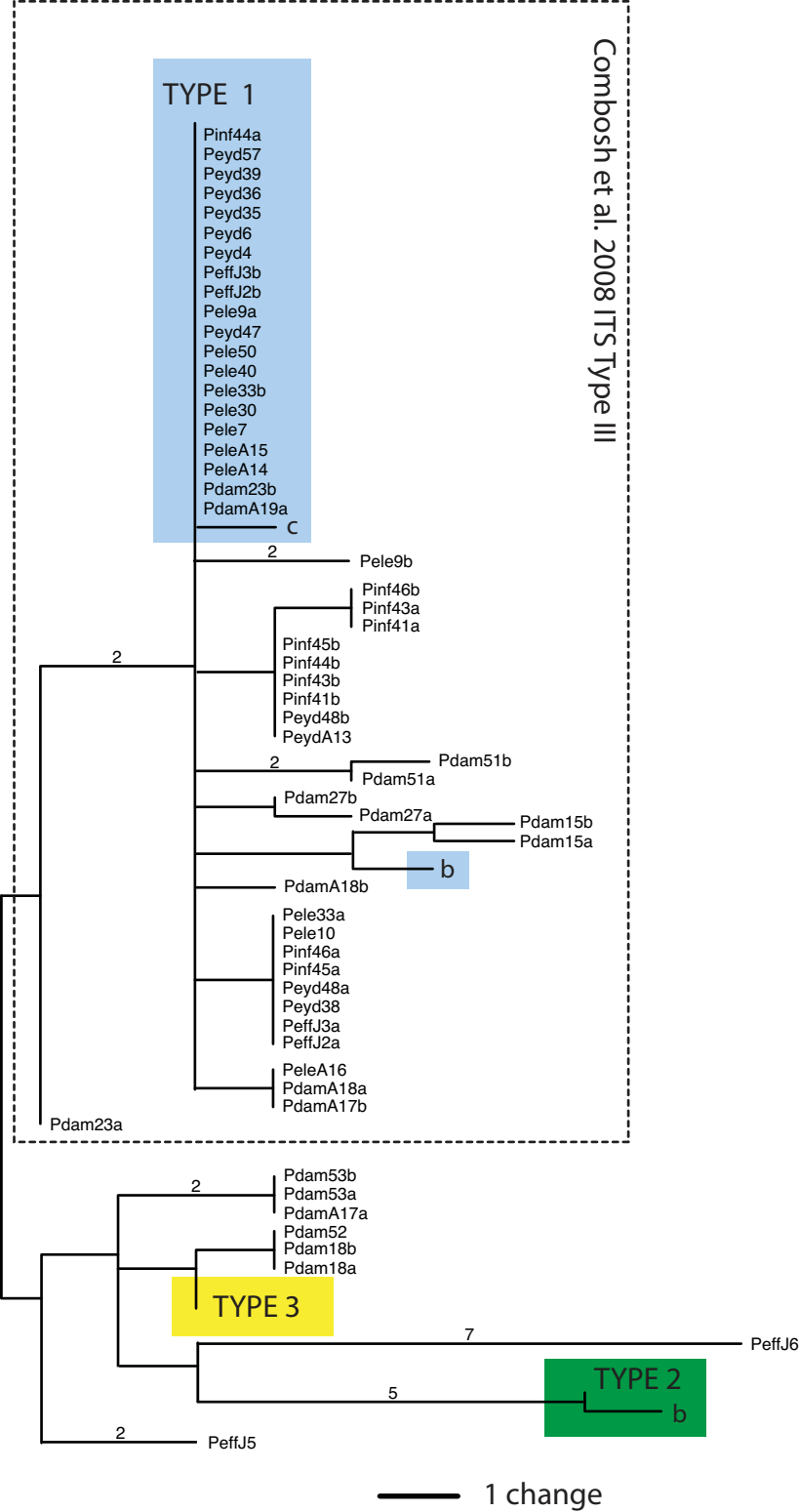
(a) Principal coordinate analysis



b. Structurama-based tree




a.
type 1
type 2
Probability of membership
K = 2
K = 3
K = 4
K = 5
Clipperton (n = 22)
b.
type 1
type 3
K = 2
K = 3
K = 4
K = 5
Panama (n = 42)


Combosh et al. 2008 ITS Type III
TYPE 1
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Peyd6
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TYPE 3
7
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TYPE 2
5
b
2
PeffJ5
1 change