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Hidden genetic diversity in a key model species of coral

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Abstract A total of 43 colonies of the scleractinian coral *Pocillopora damicornis* from lagoonal and reef slope sites in the western Indian Ocean (WIO) region were genetically characterised at one nuclear and two mitochondrial sequence markers and six microsatellite loci. Both mitochondrial and microsatellite data support the existence of two reciprocally monophyletic clusters (F- and NF-types) and provide evidence of the existence of two cryptic species of *P. damicornis* on reefs in WIO region and put current morphological delineation and geographical boundaries of *P. damicornis* and *Pocillopora molokensis* into question. The results add to ongoing studies on the phylogeny and phylogeography within the genus *Pocillopora*, which all point towards a range of unresolved morphological and molecular species boundaries. Nuclear phylogenies derived from the present and previously published sequences show evidence for incomplete lineage sorting and/or introgressive hybridisation between *Pocillopora* morphospecies. However, the two WIO types largely remain in separate clusters, further supporting the theory that these represent two different species.

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Background

Solid taxonomic units are crucial for corroborating evolutionary interpretations and support applications such as biodiversity estimates. It forms an integral part of both conservation efforts and sustainable management of habitats and is essential for testing evolutionary hypotheses. However, the accurate delineation of species by means of conventional methods and modern concepts is not always straightforward. This is mainly due to the occurrence morphologically indistinguishable (cryptic) or only a posterior distinguished (pseudo-cryptic) species (Sáez et al. 2003). Such complexes are considered rife throughout the marine realm as previously undescribed taxa are continuously being discovered within well-known taxonomic groups in our oceans (Knowlton 1993, 2000; Caputi et al. 2007).

Many coral species exhibit plastic growth forms whereby the shape of a colony varies, most commonly in response to variations in light intensity and wave movement (Todd 2008). In addition, factors such as interspecies hybridisation create a plethora of intermediate morphologies and genotypes, making species identification a daunting task (Vollmer and Palumbi 2002a). As a result, morphological and genetic delineations within and between many coral species are unsuccessful, both in massive (Miller and Babcock 1997; Miller and Benzie 1997; Lopez et al. 1999; Stobart 2000; Mangubhai et al. 2007b) and branching (van Oppen et al. 2000, 2002; Vollmer and Palumbi 2002b; Flot et al. 2008a) coral species. Indeed, the current classification of entire coral families is being questioned as new molecular data become available (Fukami et al. 2004b; Nunes et al. 2008).

Pocillopora damicornis, the target species of this study, is a common and extensively studied scleractinian coral

that is found on coral reefs throughout the world. It forms part of the family Pocilloporidae, which constitute the majority of larval recruits onto reefs in the western Indian Ocean region (Mangubhai et al. 2007a). It is hermaphroditic and can reproduce both sexually and asexually. Sexual reproduction occurs either through internal brooding of the fertilised egg (Stoddart and Black 1985) or by broadcast spawning of both egg and sperm into the water column (Ward 1992). The timing of planulation varies across its geographical range (Harriott 1983; Stoddart 1983; Richmond and Jokiel 1984; Stoddart and Black 1985; Ward 1992; Schleyer et al. 1997). Asexual reproduction occurs through the ameiotic development of the larvae (parthenogenesis) (Stoddart 1983), or through fragmentation by mechanical force or partial colony mortality.

Like many corals, the morphology of *P. damicornis* is both variable and plastic (Veron 2000; Todd 2008), ranging from finely branched in deeper waters to robust in more exposed habitats. Furthermore, it has been found to hybridise with its congeners *Pocillopora eydouxi* and *Pocillopora elegans* in the Tropical Eastern Pacific (TEP) (Combosch et al. 2008) and with its family member *Stylophora pistillata* at Lord Howe Island, Australia (Miller and Ayre 2004). As a result, the delineation of taxonomic units within this and other species within the genus *Pocillopora* remains unresolved and represents an important future challenge. Indeed, the idea of cryptic species within *P. damicornis* is not new. A potential genetic distinction between brooding and non-brooding colonies was investigated by Sherman et al. (2006). However, the study revealed no indication of reproductive barriers. The hypothesis that two cryptic species of *P. damicornis* are present in Hawaii, initially suggested by differences in morphology and reproductive timing (Richmond and Jokiel 1984), was later supported by molecular data showing that Hawaiian *P. damicornis* harbour two distinct mtDNA haplotypes (Flot et al. 2008b). Genetic delineation, both within and between morphospecies, has also been found in the TEP, where *P. damicornis* falls into three distinct types, based on the internal transcribed spacer of the nuclear ribosomal DNA (Combosch et al. 2008).

During a study of the population genetic structure of *P. damicornis* on reefs in Kenya and Tanzania, the present author revealed that all clonal lineages were homozygous for a specific allele at one of the studied microsatellite loci (PV 7). This particular allele was rare or absent in populations that showed no signs of asexual reproduction, once again raising the hypothesis of cryptic speciation (Souter et al. 2009). However, both types correspond morphologically to *P. damicornis* as shown by Fig. 1, which depicts one colony of each type. In summary, *P. damicornis* exhibits a range of characteristics that make it an ideal model species to assess species boundaries as well as the influence of hybridisation in coral cryptic speciation events.

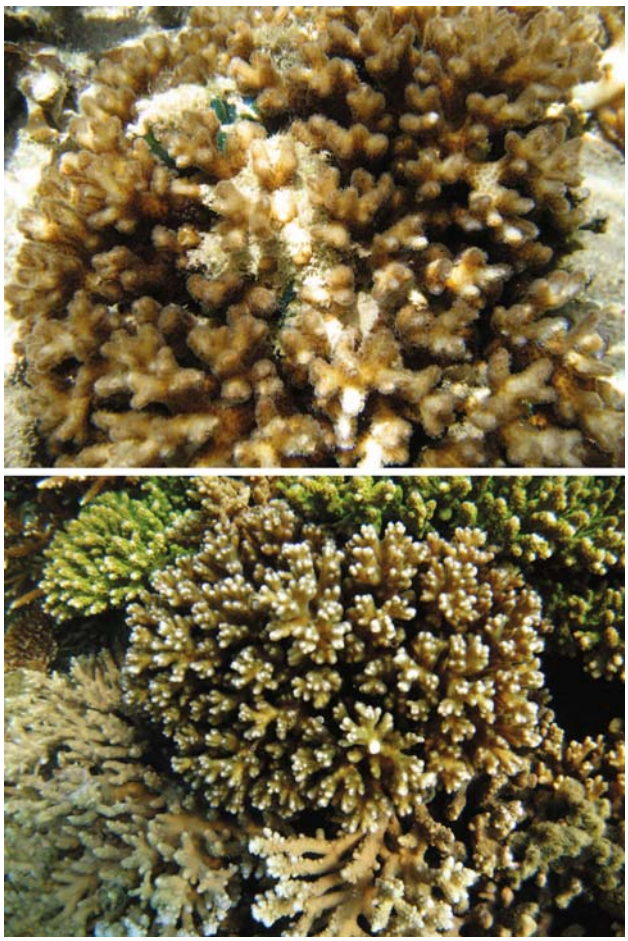

Fig. 1 Photographs depicting an F-type (*top*) and NF-type colony (*bottom*)

The aim of this study was to uncover whether cryptic genetic diversity exists within this species and to determine the level of genetic divergence between the Fixed (F-types) and Non Fixed (NF-types) PV 7 genotypes. This was undertaken by inferring phylogenies from fast-evolving nuclear and mitochondrial markers from a selection of *Pocillopora* species and geographical locations (Fig. 2). In addition, F- and NF-type colonies were genotyped at six polymorphic microsatellite loci.

Materials and methods

Samples

DNA from 26 F- and 19 NF-type colonies from 10 populations included in a previous connectivity study (Souter et al. 2009) were selected for genotyping. The colonies were randomly chosen from the data set, with at least one

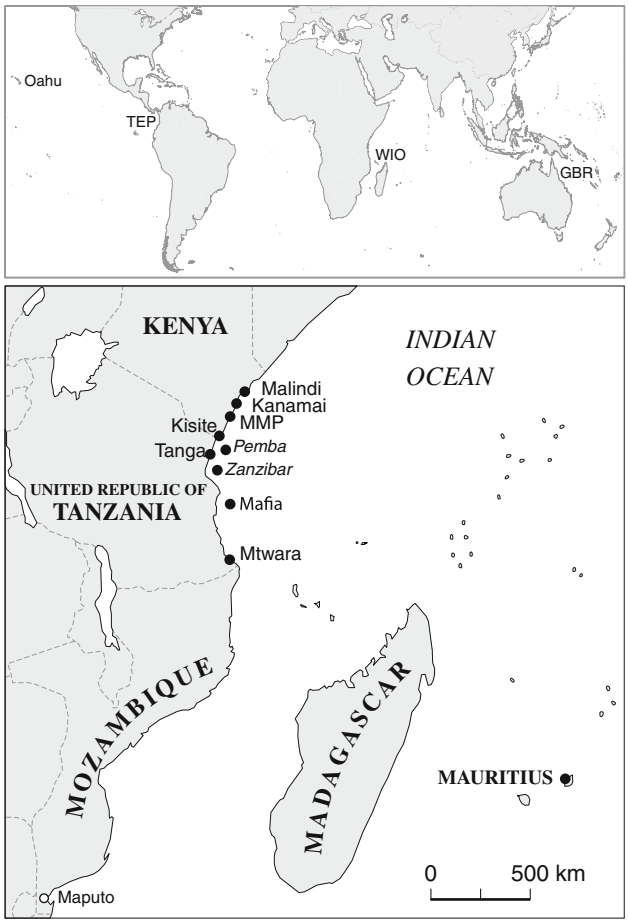

Fig. 2 Map of geographical locations included in the study

colony being selected from each sampled population. In addition, 20 F- and 4 NF-type colonies from previously unsampled sites in Kenya, Tanzania and Mauritius and four samples from the Central Great Barrier Reef were obtained and added to the data set, making a total of 73 colonies (Fig. 2).

DNA extractions and genotyping

DNA was extracted using the Qiagen® DNeasy kit according to a modified protocol for rodent tails (fragments were placed directly into lysis buffer and Proteinase K and kept in a water bath at 56°C over night). Extracted DNA was stored at −20°C.

Nuclear and mitochondrial DNA amplification and sequencing

The microsatellite locus PV7 is located within the internal transcribed spacer (ITS) 1 region of the nuclear ribosomal DNA. As the results from the microsatellite genotyping indicate that this marker may be under differential selection in the *Pocillopora damicornis* types, a fragment of the nuclear ribosomal DNA (partial 5.8S, ITS2 and partial 28S) was sequenced using the primers published by Flot and Tillier (2006). The ITS1 region was excluded as the microsatellite region proved difficult to sequence and hence produced ambiguous alignments; in addition, using the published primers allowed easy comparisons to previously published sequences of pocilloporid corals (Combosch et al. 2008; Flot et al. 2008b). Flot et al. (2008b) showed that among four tested nuclear markers (ITS2, calmodulin, elongation factor 1α and ATP synthase β subunit), the ITS region provided the most unambiguous and informative phylogeny. However, it did not resolve all intraspecific relationships. Two mitochondrial regions were amplified using the primers (ORF and CR) developed by Flot and Tillier (2007). The first consisted of partial *atp6* and an *open reading frame* (*ORF*) of unknown function and the second contained partial *nad5*, *trnW*, *atp8*, the *putative D-loop* and partial *COX 1*.

All sequencing reactions were set up in a 30 µl volume reaction containing 0.5 Scientifix Taq DNA polymerase (5 U/µl), 3 µl 10× PCR buffer (20 mM $MgCl_2$), 40 pmol of each primer and 3 µl dNTPs (2.5 mM each) and 40 ng template DNA. The thermal cycling protocol was initiated with 5 min at 95°C followed by 30× (30 s at 95°C; 30 s at 55°C (CR) or 58°C (ORF and ITS) and 1 min at 72°C) and ended with a 10-min extension at 72°C.

PCR products were purified and sequenced by Macrogen Inc., Korea, using the same primers that had been used for the PCR reaction. Ambiguous sequences, and colonies that showed genotypes indicative of being heterozygote, were cloned into chemically competent *E. coli* cells using the Invitrogen TOPO© clone kit, and four to six positive colonies were re-sequenced using the universal M13 forward and reverse primers.

Microsatellite genotyping

A polymerase chain reaction (PCR) was carried out using six fluorescently labelled microsatellite primers developed for *Pocillopora* spp: PV 6 and PV 7 (Magalon et al. 2004), and Pd3_002, Pd3_004, Pd2_006 and Pd3_005 (Starger et al. 2008). The PCR was conducted in 10 µl reactions using 25 ng of DNA, 0.25 U AmpliTaq© (Applied Biosystems) and a concentration of 0.25 mM of each dNTP, 0.1 mM of $MgCl_2$ and 0.4 mM of each primer. The thermal cycling protocol was initiated with 5 min at 95°C followed by 30× (30 s at 95°C; 30 s at 53°C (PV 6 & PV 7) or 58°C (Pd3_002, Pd3_004, Pd2_006 and Pd3_005) and 1 min at 72°C) and ended with a 10-min extension at 72°C. Non-amplifying samples were re-run at a 5°C lower annealing temperature than those stated previously. PCR products as well as positive and negative controls were

visualised on an ABI Prism 3700 DNA Analyzer (ABI, Applera Cooperation) together with a GeneScan 500-Rox ladder and genotyped automatically and verified manually using GeneMapper© version 4.0 software (ABI, Applera Cooperation).

Data analysis

Corresponding mitochondrial and rDNA sequences from 48 *Pocillopora* colonies were downloaded from the NCBI website and included in the alignments (Table 1). The two mitochondrial sequences were concatenated into a single alignment prior to further analyses. Both mitochondrial and nuclear sequences were aligned manually using MEGA ver. 3.1 (Kumar et al. 2004) and saved as nexus and Roehl format files using the programme DnaSP v 4.50.3 (Rozas et al. 2003). Roehl files were imported to the RDF editor in Network 4.1 (http://www.fluxus-engineering.com/), which was used to check for sequencing errors and ambiguous SNPs.

The best fitting evolutionary model for each alignment was estimated using the corrected Akaike information criterion (AICc) in MODELTEST v 3.07 (Posada and Crandall 1998), and a phylogeny was constructed using the inferred evolutionary constraints by the maximum likelihood calculation in PAUP 4.0b10 (Swofford 2000) using the PaupUp graphical interface (Calendini and Martin 2005). Gaps were treated as missing data, and branch support levels were estimated using 1,000 bootstrap replicates. Best trees were found using the heuristic algorithm, and sequence divergence was estimated using the Kimura's 2-parameter model. A comparative phylogeny was constructed using the maximum parsimony criteria. Resulting trees were imported into MEGA ver 3.1 where condensed trees were constructed by collapsing all branches that had bootstrap values below 0.5. All phylogenies were rooted using *Pocillopora ligulata* from Oahu as an outgroup.

Sequence divergence between groups of individuals, measured as pairwise $F_{ST,}$ was inferred from mitochondrial DNA sequences using an analysis of molecular variance (AMOVA) in a similar manner to that used for the microsatellite data, using the programme Arlequin (Laval and Schneider 2005). Comparisons were made between the two types (F and NF), geographically distant populations that fell into the same phylogenetic cluster (F and *P. damicornis* from Oahu), as well as morphologically predefined species from the same geographical location (*P. damicornis* and *P. meandrina* from Oahu).

The microsatellite tool kit (Park 2001) was used to infer allelic and genetic diversity of F- and NF-types. A genetic distance between F- and NF-type colonies, measured as pairwise population differentiation, was estimated according to Cockerham and Weir (1984) using the AMOVA framework as implemented in Arlequin (Laval and Schneider 2005). The significance value was calculated using 10,000 non-parametric permutations. This procedure is appropriate when dealing with skewed sample sizes, as it does not assume normality or equality of variance among samples. The software Genetix (Belkir et al. 1996) was used to construct a principal component analysis (PCA) to visualise the genetic distance between the F- and NF-types using each locus as an independent binary variable according to She et al. (1987).

Results

Reliable sequences were obtained from a total of 43 colonies (25 F and 18 NF), at both mitochondrial and nuclear markers. To enable a comparison of the results derived from the nuclear and mitochondrial data, only those colonies that produced reliable data from all three amplifications were selected for this study. In general, the microsatellite and mitochondrial data produced more reliable and consistent results, while the ITS sequences often degenerated across large areas of repeated sequence or showed signs of high intercolony variation. Seven of the included F-type colonies belong to three clonal lineages and were included to verify whether sequence analysis corroborates multilocus genotyping results (Table 2).

Table 1 *Pocillopora* species and geographical locations downloaded from NCBI

Morphological species	Geographical site	Accession numbers
P. damicornis	Oahu	EU 374237-238, EU 374274-276, EU374325, EU374323, EU374321
P. ligulata	Oahu	EU374250-252, EU374287-289, EU 374344-346
P. molokensis	Oahu	EU374257-259 EU374294-296, EU374360-363
P. meandrina	Oahu	EU 374281-282, EU 374244, -245, EU374336-338
P. damicornis	GBR	FJ424084-086; FJ242130-132; FJ242176-179
P. damicornis	TEP	EU314758-760
P. eydouxi	TEP	EU314770-772

Sequence analysis

Mitochondrial DNA

The concatenated mtDNA database consisted of 1,432 unambiguously aligned characters that contained 41 variable sites, of which 35 were parsimony informative (Table 3). A maximum likelihood (ML) tree, constrained with the best fitting evolutionary model (GTS), is shown in Fig. 3. *Pocillopora damicornis* specimens from Oahu and the GBR are genetically distinct and form a well-supported monophyletic clade which includes all F-type colonies. The latter is sister to a second clade formed by all NF-type colonies and *P. molokensis* from Oahu. The two *P. damicornis* types from the WIO are reciprocally monophyletic, independent of geographical location (Fig. 3). A type-specific 6-bp indel present at the mitochondrial D-loop region clearly separates the two types. However, one colony (F47) clearly clusters with the F-types, even though it contained the insertion that was otherwise unique to the NF-types. Genetic differentiation between (a) F- and NF-types sampled in sympatry, (b) geographically distant *Pocillopora damicornis* individuals and F-type colonies and (c) distinct morphological species from the same geographical location (*P. damicornis* and *P. meandrina*), measured as pairwise F_{ST} values, is significantly different from 0 ($P < 0.001$). Interestingly, the level of genetic differentiation between the F- and the NF-types is higher than between pairs of each single type and predefined morphospecies clustering in the same clade with that type. F-type colonies from the Indian Ocean and *P. damicornis* from Oahu are more similar to each other than are pairs of morphologically distinct species in Oahu (Fig. 3, Table 4).

Nuclear ribosomal DNA

The partial 5.8s—complete ITS 2—partial 28s ribosomal subunit of the nuclear ribosomal DNA region consisted of 516 aligned characters (Table 3). The best fitting evolutionary model for the ITS ML phylogeny was found to be K80, and genetic distance was measured using Kimura's 2-parameter model. ML and MP phylogenies inferred from this marker indicated the F- and NF-types of *P. damicornis* from the WIO form two genetically divergent clusters, with the exception of colony F29. The latter is found in a group of topologically unresolved species (Fig. 4). Four synapomorphic sites and an indel at the start of the ITS 2 differentiate the F-types from the NF-types with the exception of colony F29, which shows an intermediate haplotype. Subsequent cloning and sequencing of six clones carrying this amplification product indicated no evidence of it being heterozygote's as all six sequences were identical. All three *P. damicornis* sequences from Oahu are interspersed with the F-types, indicating a complete lack of divergence in the nuclear marker between these two groups. Double bases, indicative of intercolony variation (heterozygosity), were detected in only four colonies. This may be caused by the fact that the majority of the sequences were derived from direct sequencing, which has been shown to capture only part of the interindividual variation of the nuclear ribosomal DNA (Vollmer and Palumbi 2004b). None of the variable sites were parsimony informative.

Microsatellite analysis

F-types from Mauritius were found to be homozygous for allele 222 rather than 224 at locus PV7, probably as a consequence of a step mutation within these particular populations. However, both the 222 and the 224 alleles were rare or absent among the NF-types (Table 5 and Appendix 1 in electronic supplementary material). In the population genetic study of the NF-types, these two loci occurred with an average frequency of 3.2 and 2.1%, respectively, and only in heterozygote form (Souter et al. 2009). Despite there being fewer NF-types genotyped, the genetic diversity of this type was significantly greater than that of the F-types (two-tailed paired *t*-test of H_E per loci; $P = 0.012$; *df* 5) (Table 3). The level of genetic differentiation between the two types, measured as a pairwise F_{ST}, was high (0.232) and significantly different from 0 (P value < 0.0001; *df* 12). The Principal coordinate analysis plot (PCA) (Fig. 5) shows two distinct clusters of the F- and NF-types.

Discussion

Cryptic speciation

Sequence analysis and microsatellite genotyping corroborate the existence of two genetically distinct but morphologically cryptic lineages within what is currently described as *Pocillopora damicornis* in the western Indian Ocean. At times, these lineages are found in sympatry and may represent sibling species. The addition of previously obtained sequences in the mitochondrial data set shows a total of four mitochondrial lineages within this species complex, supporting the conclusions drawn by Flot et al. (2008b) that mitochondrial markers are able to resolve interspecific and intraspecific relationships among *Pocillopora* species and that sympatric cryptic lineages also exist in the Hawaiian archipelago.

The mitochondrial phylogeny further highlights the lack of congruence between morphological and phylogenetic

Table 2 Summarised information of samples from the western Indian Ocean

Colony ID	Geographical location	Microsatellite genotype						mtDNA Accession no FJ424	rDNA Accession no FJ424
		PV6	PV7	Pd3_004	Pd3_002	Pd3_005	Pd2_006		
F5 (6)	Mauritius	192 202	222 222	159 159	196 196	213 213	195 195	093; 139	215
F6 (5)	Mauritius	192 202	222 222	159 159	196 196	213 213	195 195	094; 140	204
F8	Mauritius	192 204	222 222	159 162	184 184	210 213	193 193	095; 141	221
F9	Mauritius	192 204	222 222	159 162	000 000	210 213	193 193	096; 142	219
F10	Mauritius	192 204	222 222	159 162	184 184	210 213	193 193	097; 143	218
F12	Mauritius	204 204	222 222	159 162	184 187	213 222	193 193	098; 144	205
F14 (19)	Mauritius	204 204	222 222	159 159	187 187	213 213	193 193	100; 146	206
F16	Mauritius	204 204	222 222	159 159	000 000	213 213	193 193	101; 147	207
F18	Mauritius	204 204	222 222	159 159	184 184	213 213	193 193	102; 148	208
F19 (14)	Mauritius	204 204	222 222	159 159	187 187	213 213	193 193	103; 149	209
F21	Mauritius	204 204	222 222	159 162	184 187	213 222	193 193	104; 150	216
F25	Mauritius	192 204	222 222	159 162	184 184	210 213	193 193	105; 151	222
F28	Mauritius	192 204	222 222	159 162	00000 0	210 213	193 193	106; 152	217
F29	Mauritius	193 204	222 222	159 162	000 000	210 213	193 193	107; 153	198
F31	Mauritius	192 202	222 222	159 159	196 196	213 213	195 195	108; 154	220
F32	Mauritius	192 204	222 222	159 162	187 187	210 213	193 193	109; 155	210
F38	Zanzibar	204 204	224 224	159 162	184 190	207 234	193 193	110; 156	212
F39	Malindi	192 206	224 224	159 162	184 199	213 213	197 197	099; 145	213
F41	Kanamai	192 206	224 224	159 162	184 199	213 213	197 197	092; 138	214
F42	Kanamai	192 204	224 224	159 162	184 199	216 216	197 197	091; 137	200
F43 (44,45)	Zanzibar	204 204	224 224	159 162	184 184	213 224	195 195	089; 135	203
F44 (43,45)	Zanzibar	204 204	224 224	159 162	184 184	213 224	195 195	088; 134	202
F45 (43,44)	Zanzibar	204 204	224 224	159 162	184 184	213 224	195 195	087; 133	201
F46	Zanzibar	204 204	224 224	159 162	196 199	210 224	195 195	090; 136	199
F47	Mafia	204 204	224 224	159 159	187 199	213 213	195 195	111; 157	211
NF4	Tanga	196 196	232 232	162 162	196 196	210 210	195 195	118; 164	185
NF5	Tanga	196 196	232 232	162 162	196 196	210 210	195 195	119; 165	184
NF6	Zanzibar	194 194	232 232	162 162	199 199	210 222	195 201	112; 158	187
NF7	Zanzibar	204 204	232 232	162 165	199 199	213 213	195 201	120; 166	182
NF8	MMP	208 208	230 232	162 165	196 196	210 213	195 203	126; 172	189
NF9	Pemba	000 000	230 230	162 165	196 196	216 224	187 195	125; 171	193
NF10	Mafia	204 204	230 230	162 171	196 196	210 210	195 201	113; 159	191
NF12	Zanzibar	196 204	230 230	159 159	196 196	210 210	195 195	129; 175	180
NF13	Kisite	190 204	232 232	162 162	196 205	198 213	195 197	117; 163	188
NF15	Pemba	190 202	226 230	165 168	196 196	210 213	195 195	122; 168	196
NF16	Pemba	196 206	232 232	165 165	196 196	210 210	195 195	124; 170	194
NF17	Pemba	204 204	226 226	159 162	196 196	207 210	193 203	123; 169	195
NF18	MMP	208 208	226 226	162 168	196 199	210 213	197 197	114; 160	186
NF19	MMP	196 204	230 230	165 165	187 196	210 216	195 203	128; 174	183
NF20	Mafia	194 204	230 230	162 162	000 000	207 210	195 203	121; 167	192
NF22	Zanzibar	196 198	226 232	162 168	196 199	210 213	195 197	127; 173	181
NF24	Mafia	202 206	222 230	162 165	196 196	213 216	195 201	115; 161	190
NF25	Mtwara	202 202	230 232	162 162	202 202	210 213	193 203	116; 162	197

Number in parenthesis indicates colony ID of identical genotype/s. MMP = Mombasa marine national park and reserve. Accession numbers only displayed by last three digits, all start with FJ424. Mitochondrial DNA accession numbers start with the atp6-ORF, followed by the d-loop-COX sequence number

Table 3 Diversity measures from the sequence data

	bp	*H*	N_{PS}	N_{PI}	H_D	*Pi*	*k*
mtDNA	1,432	11	41	35	0.921 (±0.006)	0.009	2.88
rDNA	516	29	31	21	0.896 (±0.029)	0.009	4.09

Size of alignment (bp), number of haplotypes (*H*), number polymorphic sites (N_{PS}), number parsimony informative sites (N_{PI}), haplotype diversity (H_D), nucleotide diversity (Pi), average number of nucleotide differences (*k*)

species, whereby the F-type clusters with what is currently considered to be *P. damicornis* but the NF-type groups more closely with *P. molokensis*. *P. molokensis* is currently described as being found only in deep waters and geographically restricted to the mid-Pacific Ocean (Veron 2000). However, the present results would indicate that the current delineation between *P. damicornis* and *P. molokensis* needs revision. This lack of congruence between morphological and phylogenetic species has been shown also in *Seriatopora* corals (Flot et al. 2008a). Other morphologically recognised species within the genus *Pocillopora* form monophyletic assemblages and are also significantly differentiated according to the AMOVA analysis. In accordance with the inferred phylogeny, the pairwise F_{ST} value between the two coexisting types from the WIO is substantially higher than that between the F-type and *P. damicornis* from Oahu. One colony (F47), which contained a 6-bp insertion otherwise unique to the NF-types, falls outside the *P. damicornis* cluster and may be the result of a hybridisation event.

Interlineage genetic variation is evident in both the F- and the NF-types. For instance, F28, F29 and F32 form a separate group of haplotypes within the F-type clade and they all originate in the same population; hence, this divergence may be correlated to geography. However, not all colonies from this particular population fall within this group. Moreover, the two clusters within the NF-types show no apparent phylogeographic pattern, indicating that the mitochondrial marker may not adequately resolve intraspecies population boundaries at smaller geographical scales.

Regarding the ensuing large scale phylogeographic patterns, it is interesting to note that the four distinct mitochondrial lineages within *P. damicornis* correspond to broad geographical locations, with the exception of one Oahu haplotype, which groups with samples from the GBR. Evidence of multiple species of *P. damicornis* has previously been reported from Hawaii, based both on reproductive differences (Richmond and Jokiel 1984) and the presence of divergent mitochondrial haplotypes (Flot et al. 2008b). It may thus be possible that the previously encountered mitochondrial lineages are derived from past or recent migrants from the South Pacific and may represent distinct biological species. This is further supported

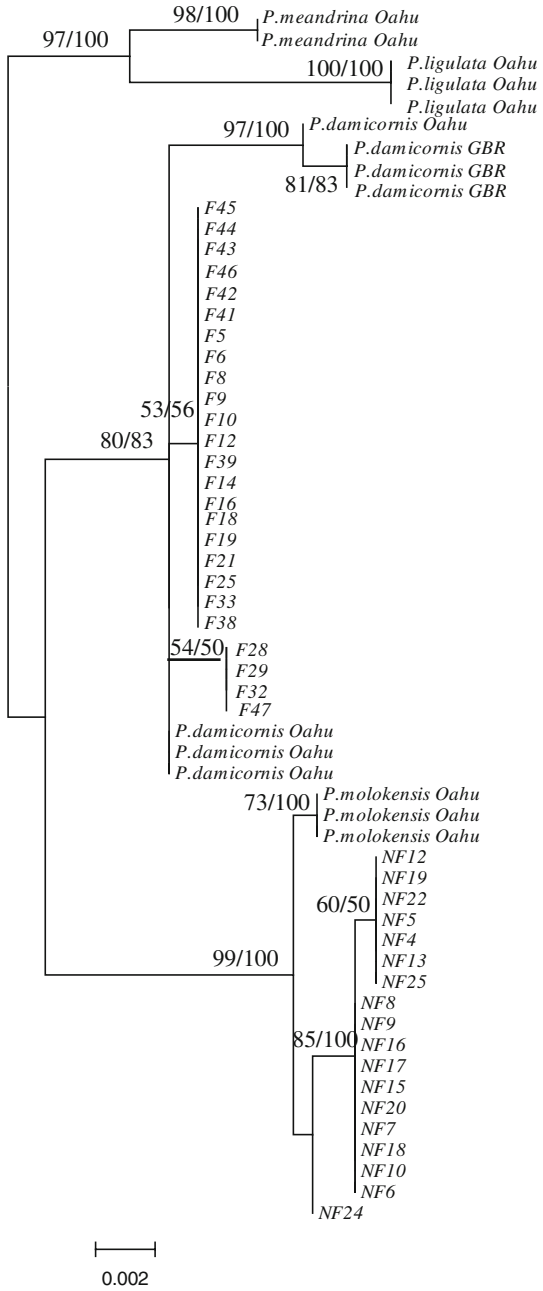

Fig. 3 Bootstrap maximum likelihood phylogeny of concatenated mtDNA sequences. The phylogeny is condensed to show only branches supported by bootstrap values over 50%. Bootstrap values for the maximum parsimony criteria also shown MP/ML

by differences in the lunar periodicity of reproduction (Richmond and Jokiel 1984). Migration across the Pacific by means such as rafting has been touted as a possibility (Jokiel 1984), and *Pocillopora* species are among the most commonly encountered rafting species in the Pacific (Jokiel 1989).

The low bootstrap values that separate the two types in the rDNA phylogeny, coupled with the position of colony F29, indicates that the ITS marker is not suitable to

Table 4 Pair wise F_{ST} values, calculated from concatenated mitochondrial DNA sequence data using the software Arlequin

Pair wise comparison	F_{ST}	P value
F/NF	0.939	0.000
F/Oahu *P. damicornis*	0.351	0.000
Oahu *P. damicornis*/Oahu *P. meandrina*	0.919	0.000

Values were measured between lineages sampled at the same geographical location (F and NF), geographically distant groups that share a common ancestral node (F and *P. damicornis* at Oahu) and morphological species at the same geographical location (*P. damicornis* and *P. meandrina* at Oahu)

distinguish the two types. As with many other nuclear markers, the ribosomal DNA region is plagued by high intraspecific and intraindividual diversity in corals. In *Acropora aspera*, up to nine rDNA copies have been found in a single colony (van Oppen et al. 2002), and divergence among sequences within a single colony can be as high as 11.4% in the ITS 1 region and 3.8% in the 18s ribosomal gene (Vollmer and Palumbi 2004a). Much of this is thought to be the result of incomplete lineage sorting due to slow convergent evolution of unsorted ancestral polymorphisms, introgression between hybrid species (van Oppen et al. 2000, 2001; Combosch et al. 2008) and the existence of non-functional pseudo-genes of nuclear ribosomal DNA (Marquez et al. 2003). Conversely, a study of intraindividual and interindividual variation in the ITS 1 region of the pocilloporid coral *Seriatopora hystrix* showed that only 2% of the observed variation is intraindividual (Zvuloni et al. 2008), and in the study by Combosch et al. (2008), no more than two alleles of the ribosomal DNA marker were found in any one individual. In this study, obtaining good sequencing data from the ITS 2 region proved the most difficult. This difficulty may be attributed to a range of factors, including the existence of multi-copy genes and/or pseudo-genes, large intraindividual polymorphisms, hybridisation or further speciation. However, the 5.8s and 28s genes are highly conserved and phylogenetically uninformative in the *Pocillopora*, and the removal of the ITS 2 from the alignment created an entirely unresolved phylogeny (data not shown).

The other nuclear markers, the microsatellites, show a clear differentiation, indicating a lack of or very limited gene flow between the two types. The pairwise differentiation between the F- and NF-types ($F_{ST} = 0.232$) can be put into context when compared with the degree of differentiation that has been reported between populations of the NF-type ($F_{ST} = 0.023$), which was derived using the same markers and the same geographical locations (Souter et al. 2009). This distinct delineation remains also when

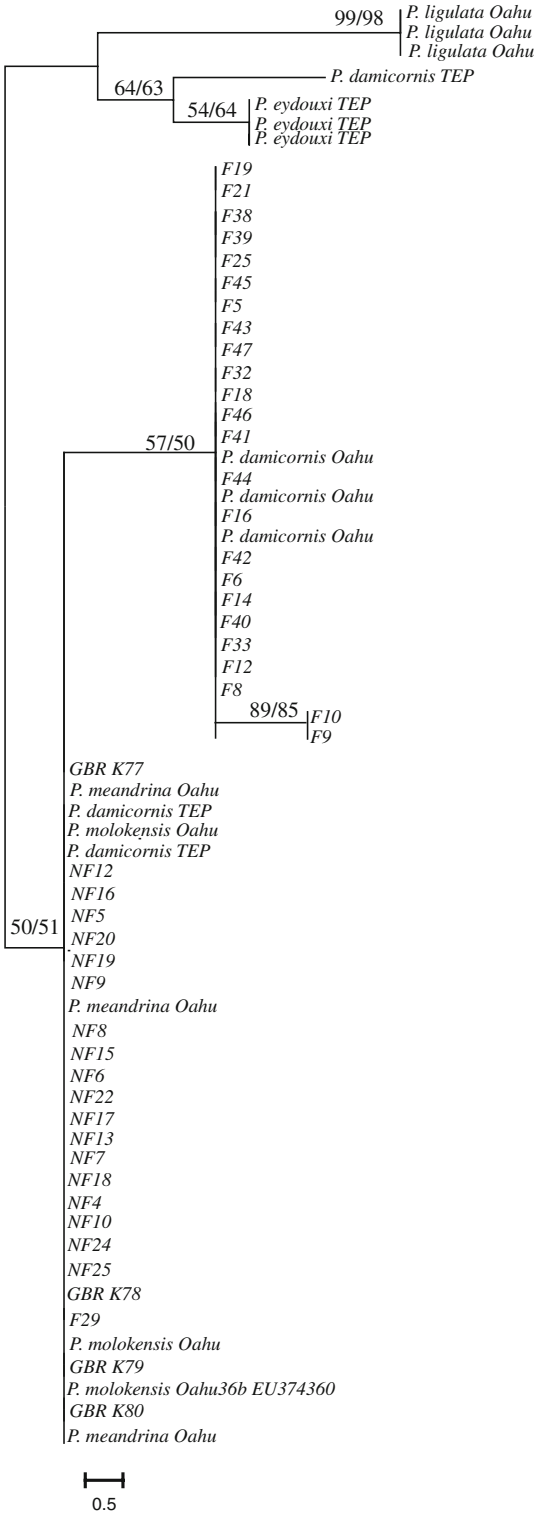

Fig. 4 Bootstrap maximum likelihood phylogeny of partial 5.8s full ITS 2 and partial 28s nuclear rDNA. The phylogeny is condensed to show only branches supported by bootstrap values over 50%. Bootstrap values for the maximum parsimony criteria also shown MP/ML

Table 5 Genetic diversity of the F and NF clusters at the six microsatellite locus

	N	H_E	H_O	*a*
F	46	0.534 (±0.048)	0.354 (±0.030)	4.17 (±2.32)
NF	23	0.711 (±0.035)	0.463 (±0.043)	7.00 (±2.53)

Number of genotyped individuals (*N*), Nei's unbiased genetic diversity measured as expected heterozygosity (H_E), observed heterozygosity (H_O) and average number of alleles per loci (*a*) (±SD)

using all 825 colonies that were originally genotyped at the microsatellite loci for the population genetic study. It is thus unlikely that the failed ITS sequences constitute hybrids between the two types, nor represent an additional cryptic species as this would have been apparent also in the microsatellite data.

The topological incongruence between mitochondrial and nuclear phylogenies suggests incomplete lineage sorting and extensive hybridisation among the four mitochondrial lineages. However, both phylogenies are mostly congruent in their delineation of the F- and NF-types, something that strongly supports the theory of two reproductively isolated species within *P. damicornis*. The results indicate that F- and NF-types do not undergo hybridisation and may represent the only biologically distinct species within the *Pocillopora damicornis* species complex uncovered to date. Furthermore, both phylogenies group the NF-types with *P. molokensis* albeit this cluster remains unresolved for three species and four geographical regions in the ITS phylogeny. Combosch et al. (2008) inferred three ITS types (I, II and III) among *Pocillopora* corals in the tropical eastern and central western Pacific. The present phylogeny does not fall into any apparently comparable clusters, and neither the F- nor the NF-types are grouped with the TEP type III *Pocillopora damicornis*. While introgressive hybridisation is the most likely explanation for shared interspecies haplotypes in the TEP (Combosch et al. 2008), the idea of such hybridisation causing the lack of monophyletic groups in this study seems less plausible due to the large geographical distances and lack of geographical clustering. The present data set is not based on exhaustive sequencing and sampling to uncover all potential alleles of the ITS region, hence making an accurate prediction regarding the likelihood of introgressive hybridisation is not possible.

A significantly lower proportion of identical multi-locus genotypes in the NF-types than among the F-types was found in the population genetic data (Fisher's exact test: $P < 0.01$). In total, 105 colonies out of 154 were found to belong to 14 clonal lineages in the F-types, while among the NF-types, only a single identical multi-locus genotype was encountered among 661 samples (Souter et al. 2009). This dual mode of reproduction in *P. damicornis* has been highlighted in studies from the GBR, where sexual reproduction was dominant in adult populations despite the fact that all brooded planulae were genetically identical to their brood parent (Ayre and Miller 2004; Sherman et al. 2006). Furthermore, a mix of brooding and broadcast spawning, occurring at different times of the year, has been reported from Western Australia (Ward 1992). However, it is not evident from the results if the broadcast spawning colonies were the same colonies as those producing brooded larvae. Such a difference in reproductive mode may explain the lower genetic diversity of the F-type colonies. Many earlier

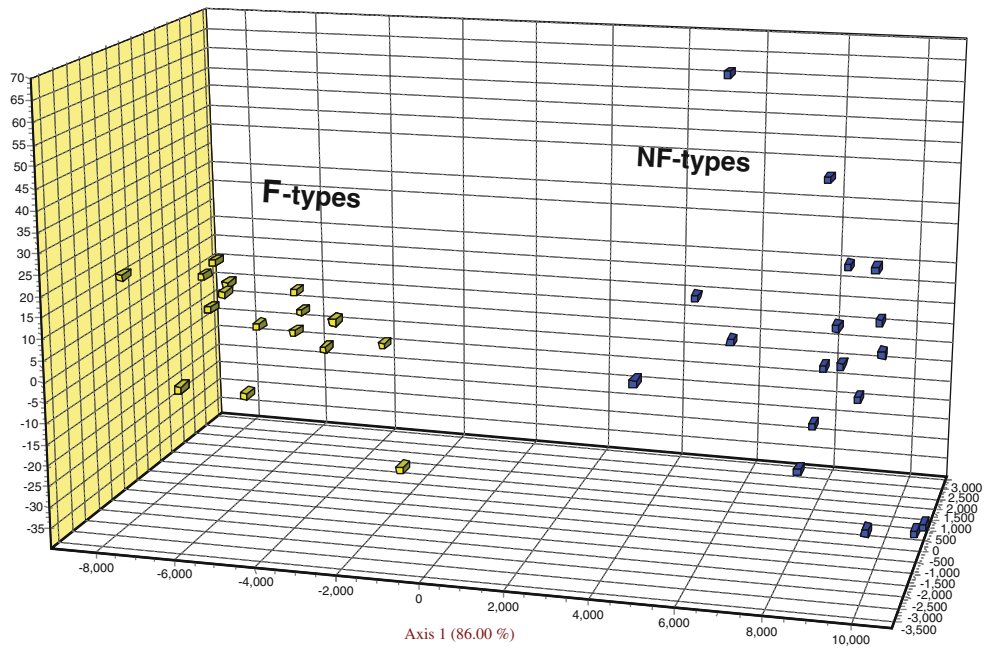

Fig. 5 Principal component analysis of F (*yellow*) and NF (*blue*) microsatellite genotypes

studies of *P. damicornis* have revealed a variable impact of asexual reproduction between sampled populations (Stoddart 1984a, b, 1986; Benzie et al. 1995; Ayre et al. 1997; Adjeroud and Tsuchiya 1999; Miller and Ayre 2004; Whitaker 2006). To date, populations dominated by identical multi-locus genotypes have only been recorded from reefs that can be considered disturbed (Sherman et al. 2006) or geographically marginal (Stoddart 1983, 1984a, 1986). However, not all disturbed or geographically marginal habitats show a prevalence of asexual reproduction, and there is no evidence that a correlation between habitat or disturbance and reproductive mode exists (Miller and Ayre 2004).

The extent of the genetic divergence between these two types on reefs in the WIO, and the fact that the F-types are more closely related to *P. damicornis* from distant geographical locations than to their "conspecifics" that were sampled on the same reef, indicates deep separation and a lack of gene flow between these types on an evolutionary time scale rather than one caused by current selection. The fact that these two cryptic species may coexist throughout the geographical range of the species complex coupled with increasing evidence of polyphyletic clades within *Pocillopora* (Combosch et al. 2008; Flot et al. 2008b) calls for caution in the interpretation of some of the results from previous studies. In addition, it is worth questioning the assumption that all these studies are based on the same species, which is something a more thorough phylogeographic study would resolve.

As with many other taxonomic groups of corals, such as the *Acropora aspera* group (van Oppen et al. 2002) and the *Montastraea annularis* group (Lopez et al. 1999; Fukami et al. 2004a), the *Pocillopora* corals show a complex pattern of morphological and phylogenetic species boundaries. Not surprisingly, much of the emerging molecular data is revealing a far greater diversity in corals than previously believed and, as a result, conventional taxonomic classification of corals is currently in question at the family (Fukami et al. 2004b; Nunes et al. 2008), to genera (Miller and Babcock 1997; Miller and Benzie 1997; van Oppen et al. 2001, 2002; Mangubhai et al. 2007b; Forsman et al. 2009) and down to the species levels (Stobart 2000; van Oppen et al. 2000). More often than not a clear link between genetic and morphological delineations is lacking, indicating that basing the taxonomy of corals on skeletal characters alone leads to inaccurate and more importantly underestimated predictions of the diversity of this order.

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