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## Patterns of evolution in the scleractinian coral genus *Montipora* (Acroporidae)

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**Abstract** One of the key issues in coral evolutionary biology is the significance of interspecific hybridisation in shaping modern reef corals. Despite this, few detailed studies of speciation and evolution exist for scleractinians. We examined molecular relationships among 25 species of the species-rich scleractinian genus *Montipora* sampled from Irian Jaya (Indonesia) and Magnetic Island (central Great Barrier Reef) using a nuclear and mitochondrial marker. Mitochondrial DNA (mtDNA) haplotypes are shared among several species and at least four species are not monophyletic based on the nuclear intron sequences. Moreover, although the mtDNA and nuclear trees have similar topologies, there are several important inconsistencies. These results, in combination with the observation of simultaneous spawning of and in vitro cross-fertility between some *Montipora* species, suggest that occasional gene exchange through interspecific hybridisation occurs between a small number of species in this coral genus. Little correlation was observed between morphological and molecular relationships.

### Introduction

The prevalence of external fertilization in the sea suggests that the mating systems of many marine organisms

may be conducive to hybridisation (Gardner 1997). The highly synchronized spawning of many sympatric, congeneric species of corals (Harrison et al. 1984; Willis et al. 1985; Babcock et al. 1986) provides the most obvious opportunity for hybridisation in the marine realm. At least 133 species of corals from ten scleractinian families are involved in mass spawnings over four to five nights annually on the Great Barrier Reef (GBR; Willis et al. 1985). Multispecific spawnings, generally involving smaller numbers of species, also occur in other reef regions (Heyward et al. 1987; Simpson et al. 1991; Hayashibara et al. 1993; Babcock et al. 1994; de Graaf et al. 1999), including equatorial reefs (van Veghel 1993; Sánchez et al. 1999; Guest et al. 2002). Experimental breeding trials have demonstrated that co-occurring, congeneric species are able to interbreed with high fertilization success, suggesting that hybridisation can occur and is likely to have contributed to the evolution of scleractinian corals (Willis et al. 1997; Wallace and Willis 1994; Miller and Babcock 1997; Szmant et al. 1997; Hatta et al. 1999).

Of the coral genera found on Indo-Pacific reefs, *Acropora* is by far the most species-rich with at least 180 morphological species (Veron 2000), followed by its family member *Montipora* with 75 morphological species (Veron and Wallace 1984; Veron 2000). *Montipora* is widely distributed, spanning the Indian and Pacific Oceans, but is absent in the Atlantic. Colony morphology in this genus varies from submassive to laminar, encrusting and branching colonies (Veron and Wallace 1984; Veron 2000). The molecular relationships and evolutionary history of *Acropora* species are currently being disentangled by several research groups (Odorico and Miller 1997; Hatta et al. 1999; van Oppen et al. 2000, 2001, 2002b; Márquez et al. 2002a, 2002b; Vollmer and Palumbi 2002), but similar studies are scarce for *Montipora*, with only two published studies so far. Allozyme electrophoresis shows that, on Magnetic Island in the central GBR, *M. digitata* consists of two genetically distinct populations that also differ slightly in morphology (Stobart and Benzie 1994; Stobart 2000).

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Using mitochondrial DNA (mtDNA) Cytb and ATP6, Fukami et al. (2000) indicated that *M. aequituberculata* is more distantly related to *M. digitata*, *M. altasepta*, and *M. efflorescens* than to *Anacropora* species, although the latter is a distinct genus showing little microskeletal variation. In *Acropora*, morphological and molecular relationships are largely incongruent (van Oppen et al. 2001). Moreover, many species are not monophyletic and several important differences exist between nuclear and mitochondrial phylogenies. Species that do form monophyletic groups tend to differ in the timing of release of their gametes. These results, in combination with studies on cross-fertility (Willis et al. 1997) and population genetic approaches (Márquez et al. 2002a, 2002b), suggest that during mass spawning *Acropora* species in the Indo-Pacific cross-fertilize at an unknown frequency, resulting in at least some interspecific gene exchange. Moreover, of the three *Acropora* species that occur in the Caribbean, *A. prolifera* is the outcome of hybridisation between *A. palmata* and *A. cervicornis* (van Oppen et al. 2000; Vollmer and Palumbi 2002).

Although the evidence for reticulation in *Acropora* is now compelling, the extent to which this applies to other genera is not clear. Nevertheless, studies on two non-acroporid coral genera belonging to different families support the occurrence of natural introgressive hybridisation. Allozyme divergence between three of the seven recognized *Platygyra* morphospecies (Favidae) on the GBR is low (Nei's *D* range from 0.032 to 0.057) and preliminary electrophoretic screening of the other four species suggests that this pattern is consistent throughout the genus (Miller and Benzie 1997).  $F_{st}$  values at five of the nine loci tested were significantly different from zero and mean  $F_{st}$  values among morphospecies at Davies Reef were low (0.053), but significantly different from zero (note that sample sizes were small; Miller 1994). Moreover, all species spawn synchronously and experimental breeding trials show that morphological species are highly cross-fertile (Miller and Babcock 1997). In the Caribbean brooding coral *Madracis* (Pocilloporidae), high levels of morphological plasticity occur among the five most common sympatric species. Temporal reproductive isolation is absent in the genus, with mature gametes being present in these brooding species in autumn (August–November; Vermeij 2002). Three of the five species share ribosomal DNA Internal Transcribed Spacers (rDNA ITS) sequences (Diekmann et al. 2001). For one of these species, *M. decactis*, the oldest known fossils are 15–11 million years old (Budd et al. 1994; Swedburg 1994); *M. pharensis* is of more recent origin [1.5 million years (Budd and Johnson 1999)]; and no fossils are known for *M. formosa*. Given the observed sequence divergence within the genus (approximately 6%) and approximate age of the species, homogenisation of ITS regions would normally be expected. Hence, the non-monophyly of the three species suggests that they exchange genes through introgressive hybridisation (Diekmann et al. 2001).

Here we examine molecular relationships among 25 *Montipora* species, representing 10 of the 12 growth groups defined based on a combination of growth form and skeletal characters (Veron 2000), from Indonesia and the Great Barrier Reef. Two molecular markers are used, the putative control region of the mitochondrial genome and the *Pax-C* 46/47 intron in the nuclear genome. These have previously been applied to reconstruct a phylogeny of species in the genus *Acropora* (van Oppen et al. 2001). The *Pax-C* gene is a homeobox gene and is single-copy in most organisms, including acroporid corals (Callaerts et al. 1997; Catmull et al. 1998; van Oppen et al. 2001). The putative control region in *Acropora* is located in between the small subunit rDNA gene (*rns*) and *cox3* (van Oppen et al. 2002a), and a large non-coding region is present at the same location in the *Montipora* mtDNA. It is assumed that this region is homologous to the putative control region in *Acropora* and is referred to as the control region in the remainder of the text. Thus, this study addresses the relationships between morphological species in the scleractinian genus *Montipora* determined from both nuclear and mitochondrial DNA and examines the possible occurrence of interspecific hybridisation.

## Materials and methods

### Collection of samples

A small piece (2–5 cm) of living material was snapped off individual colonies and preserved in 90–100% ethanol. An additional larger piece was collected and bleached for identification. Samples were collected from Irian Jaya, Indonesia (V-series) and Magnetic Island, central Great Barrier Reef, Australia (M-series; Table 1) and identified by Veron. All sites in Irian Jaya were within about 50 km of each other, except for those 'from the same locality' which were within 20 m of each other, on the same patch of reef.

### DNA extraction and PCR amplification

DNA was extracted from a tiny piece of ground-up coral using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol. DNA was eluted from the Qiagen column twice with 150 µl elution buffer. The *Acropora rns*\_FP1 and *cox3*\_RP1 primers (van Oppen et al. 2001) were used to amplify the mitochondrial control region. One microlitre of undiluted DNA (second eluate) was added to 24 µl of PCR master mix consisting of 1 µl of each primer at a concentration of 10 µM, 2.5 µl dNTPs (2 mM), 2.5 µl 10x reaction buffer (Fisher Biotech), 2 µl MgCl<sub>2</sub> (25 mM), 0.13 µl Taq polymerase (5.5 um/µl; Fisher Biotech) and 14.87 µl H<sub>2</sub>O. The PCR profile was an initial denaturation step of 3 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Finally, the mixture was incubated at 72°C for 5 min. Two additional primers (ms\_FP2:5'-TAGACAGGGCCAAGGAGAAG-3' and MON\_RP2:5'-GATAGGGGCTTTTCATTTGTTT-3') were designed based on the *Montipora* control region sequences obtained early on in this study, as some samples did not amplify efficiently using *rns*\_FP1 and *cox3*\_RP1. The *Pax-C* intron was initially amplified in *Montipora* using the *Acropora* primers *PaxC*\_intron-FP1 and *PaxC*\_intron-RP1 (5'-GGCGATTTGA-GAACCAAACCTGTA-3'; van Oppen et al. 2001), the PCR was product cloned and sequenced, and a new *Montipora*-specific forward primer was designed (Mont\_Pax-FP1:5'-GGATGTAGC-CACGAGAGAG-3').

**Table 1** Sample codes, collection locations, and sequences obtained for samples used in this study. *CR* control region; *Pax Pax-C* intron; *I.J.* Irian Jaya (Indonesia)

| Sample code | Species                     | Collection location             | Sequences obtained (No. of clones sequenced) |
|-------------|-----------------------------|---------------------------------|--|
| V50         | <i>Montipora sp.1</i>       | I.J.                            | CR, Pax (3)                                  |
| V85         | <i>Montipora sp.2</i>       | I.J.                            | CR, Pax (2)                                  |
| V35         | <i>M. altasepta</i>         | I.J., same locality as V45, V53 | CR, Pax (2)                                  |
| V45         | <i>M. altasepta</i>         | I.J., same locality as V35, V53 | CR, Pax (1)                                  |
| V53         | <i>M. altasepta</i>         | I.J., same locality as V35, V45 | CR, Pax (1)                                  |
| V8          | <i>M. angulata</i>          | I.J., same locality as V39, V70 | CR, Pax (2)                                  |
| M342        | <i>M. aequituberculata</i>  | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M343        | <i>M. aequituberculata?</i> | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| V22         | <i>M. cactus</i>            | I.J., same locality as V31, V71 | CR, Pax (1)                                  |
| V31         | <i>M. cactus</i>            | I.J., same locality as V22, V71 | CR, Pax (2)                                  |
| V60         | <i>M. cactus</i>            | I.J.                            | CR, Pax (2)                                  |
| V71         | <i>M. cactus</i>            | I.J., same locality as V22, V31 | CR, Pax (1)                                  |
| V16         | <i>M. capitata</i>          | I.J., same locality as V40, V95 | CR, Pax (1)                                  |
| V39         | <i>M. capitata</i>          | I.J., same locality as V8, V70  | CR, Pax (1)                                  |
| V40         | <i>M. capitata</i>          | I.J., same locality as V16, V95 | CR   |
| V70         | <i>M. capitata</i>          | I.J., same locality as V8, V39  | CR, Pax (1)                                  |
| V95         | <i>M. capitata</i>          | I.J., same locality as V40, V16 | CR, Pax (2)                                  |
| V77         | <i>M. capricornis</i>       | I.J.                            | CR, Pax (2)                                  |
| V3          | <i>M. confusa</i>           | I.J.                            | CR, Pax (1)                                  |
| V9          | <i>M. confusa</i>           | I.J.                            | CR, Pax (3)                                  |
| V29         | <i>M. confusa</i>           | I.J.                            | CR, Pax (3)                                  |
| V89         | <i>M. confusa</i>           | I.J.                            | CR, Pax (1)                                  |
| V11         | <i>M. danae</i>             | I.J., same locality as V23, V30 | CR, Pax (2)                                  |
| V23         | <i>M. danae</i>             | I.J., same locality as V11, V30 | CR, Pax (2)                                  |
| V30         | <i>M. danae</i>             | I.J., same locality as V11, V23 | CR, Pax (2)                                  |
| V37         | <i>M. danae</i>             | I.J.                            | CR, Pax (2)                                  |
| V1          | <i>M. delicatula</i>        | I.J., same locality as V25, V41 | CR, Pax (1)                                  |
| V25         | <i>M. delicatula</i>        | I.J., same locality as V1, V41  | CR, Pax (2)                                  |
| V41         | <i>M. delicatula</i>        | I.J., same locality as V1, V25  | CR, Pax (2)                                  |
| V63         | <i>M. digitata</i>          | I.J.                            | CR, Pax (1)                                  |
| V7          | <i>M. florida</i>           | I.J., same locality as V79, V86 | CR, Pax (3)                                  |
| V79         | <i>M. florida</i>           | I.J., same locality as V7, V86  | CR, Pax (1)                                  |
| V86         | <i>M. florida</i>           | I.J., same locality as V7, V79  | CR, Pax (2)                                  |
| V82         | <i>M. gaimardi</i>          | I.J.                            | CR, Pax (1)                                  |
| V4          | <i>M. hispida</i>           | I.J., same locality as V34, V62 | CR, Pax (1)                                  |
| V34         | <i>M. hispida</i>           | I.J., same locality as V4, V62  | CR, Pax (2)                                  |
| V62         | <i>M. hispida</i>           | I.J., same locality as V4, V34  | CR, Pax (1)                                  |
| V78         | <i>M. hispida?</i>          | I.J.                            | CR, Pax (1)                                  |
| M331        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M334        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M336        | <i>M. hispida?</i>          | Nelly Bay, Magnetic Island      | CR, Pax (1)                                  |
| M337        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M338        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M340        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (1)                                  |
| M341        | <i>M. hispida</i>           | Nelly Bay, Magnetic Island      | CR, Pax (3)                                  |
| V42         | <i>M. hoffmeisteri</i>      | I.J., same locality as V47, V88 | CR, Pax (1)                                  |
| M349        | <i>M. mollis</i>            | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M339        | <i>M. peltiformis</i>       | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M346        | <i>M. peltiformis</i>       | Nelly Bay, Magnetic Island      | CR, Pax (2)                                  |
| M350        | <i>M. spongodes</i>         | Nelly Bay, Magnetic Island      | CR, Pax (3)                                  |
| V10         | <i>M. stellata</i>          | I.J.                            | CR, Pax (2)                                  |
| V14         | <i>M. stellata</i>          | I.J., same locality as V18      | CR, Pax (1)                                  |
| V18         | <i>M. stellata</i>          | I.J., same locality as V14      | CR   |
| V52         | <i>M. turtlensis</i>        | I.J.                            | CR, Pax (2)                                  |
| V2          | <i>M. undata</i>            | I.J.                            | CR, Pax (1)                                  |
| V12         | <i>M. undata</i>            | I.J.                            | CR, Pax (1)                                  |
| V15         | <i>M. verrucosa</i>         | I.J.                            | CR, Pax (1)                                  |
| V33         | <i>M. verrucosa</i>         | I.J.                            | CR, Pax (2)                                  |
| V72         | <i>M. verrucosa</i>         | I.J.                            | CR, Pax (1)                                  |

#### Cloning and sequencing

PCR products were mixed with 5 µl orange G loading dye and loaded on a 1% agarose-TAE gel, along with a 1-kb size marker. PCR products were excised from the gel and spun through a no. 1 Whatman paper filter for 1 min. at 5,000 rpm in a bench-top Eppendorf centrifuge. These purified products were either sequenced

directly (control region) or cloned into a pGEM-T and pGEM-T Easy Vector Systems (Promega; *Pax-C* intron, as some individuals are heterozygous). Inserts were amplified from white colonies using vector primers, run on an agarose gel, and spun through Whatman paper as described above. Products were quantified and sequenced on an ABI 310 genetic analyser (Perkin Elmer) or a CEQ2000 sequencer (Beckman Instruments Inc.).

## Data analysis

Sequences were proofread and aligned in Sequencher 4.1 (Gene Codes Corporation). Maximum likelihood (ML) analyses were performed in MolPhy 2.3 (Adachi and Hasegawa 1996) under the HKY85 model of sequence evolution. Transition/transversion (ts/tv) ratios were estimated from the data. Neighbour-joining distance and parsimony (heuristic search) analyses were conducted in PAUP\* 4.0b10 (Swofford 1999). Bootstrap analysis was performed with 1,000 replicates whenever possible, or fewer replicates if computing time became excessive. Pairwise sequence distances were calculated in PAUP\* 4.0b10. Analyses under a constraint topology were carried out using the Shimodaira and Hasegawa tests of phylogenetic hypotheses (Shimodaira and Hasegawa 1999) in SHTests v1.0 (written by Andrew Rambout) to examine statistical significance of the difference in tree topologies.

## Results

### Base composition, genetic distances, and saturation of *Montipora* sequences

Base compositions of the control region and the *Pax-C* intron are shown in Table 2. The control region has a bias toward G and T and strongly away from C. The *Pax-C* intron has a high A and T content. Maximum corrected pairwise sequence distances within *Montipora* are higher for the nuclear intron than for the mtDNA intergenic region (Table 3), due to low substitution rates in anthozoan mtDNA (van Oppen et al. 1999; Shearer et al. 2002). The ts/tv ratio is 2.1 for the *Pax-C* intron and 3.4 for the control region, indicating that sequence saturation is not an issue for either of the data sets.

### Control region

The alignment of the mitochondrial control region consists of sequences from 59 coral colonies, 46 sequences from the V-series and 13 sequences from the M-series (GenBank accession numbers AY313547–AY313605). The control region alignment consists of

**Table 2** Mean base compositions (percent) of the *Pax-C* intron and control region in *Montipora* species

|                     | A    | C    | G    | T    |
|---------------------|------|------|------|------|
| Control region      | 22.8 | 12.8 | 27.0 | 37.4 |
| <i>Pax-C</i> intron | 30.9 | 20.0 | 20.7 | 28.4 |

**Table 3** Range of Kimura two-parameter pairwise sequence distances (percent) for the *Pax-C* intron and the mtDNA control region among and within *Acropora* (28 species) and *Montipora* (22 species, excluding the largely unalignable alleles found in *M. delicatula* V25.1 and *M. hoffmeisteri* V42.2) species, respectively

|                  | <i>Pax-C</i> intron | mtDNA control region |
|------------------|---------------------|----------------------|
| <i>Acropora</i>  | 0.00–13.90          | 0.00–6.90            |
| <i>Montipora</i> | 0.00–5.91           | 0.00–1.72            |

536 positions, of which 5 positions are variable (but not parsimony informative) and 20 are parsimony informative (gaps excluded). The total control region consists of 638 positions, but approximately 100 bp of the 5'-end of the control region are missing as the *Montipora*-specific forward primer (ms-FP2) anneals within the control region. Nineteen haplotypes are present among the 59 sequences based on ML analysis, whereas statistical parsimony (as implemented in TSC version 1.13; Clement et al. 2000) distinguishes only 16 haplotypes. A blast search revealed that a segment of the control region, consisting of 69 nucleotides, has an 88% similarity with a coding part of the mitochondrial *cox3* region of *Acropora tenuis* (Fig. 1). This region is highly conserved in *Montipora*; no variable sites are present within the *Montipora* sequences presented here. The mechanism responsible for the origin of this duplicated segment remains to be elucidated. In contrast to the *Acropora* control region, which has a considerable number of repeat regions (van Oppen et al. 2002a), no other repeat regions were observed in the *Montipora* control region.

The likelihood ratio test (as implemented in the program Modeltest (Posada and Crandall 1998) revealed that the Hasegawa–Kishino–Yano (HKY85) model has the best fit to the control region data set. A ML tree, based on the control region alignment, was constructed using the HKY85 model in the program MolPhy (Adachi and Hasegawa 1996; Fig. 2A). The tree is unrooted, because no suitable outgroups were available. Maximum parsimony (MP) yielded the same results; the consensus tree of the five most parsimonious trees (gaps excluded) resulted in the same topology (tree length = 26) with slightly lower bootstrap values (data not shown). A neighbour-joining analysis also resulted in an identical topology. Based on clustering pattern of taxa and bootstrap values, seven clades can be distinguished. The first clade comprises five species (*M. capitata* V39 and V70, *M. angulata* V8, *M. undata* V2 and V12, *M. gaimardi* V82, and *M. confusa* V3). The second small clade only contains one sample of *M. confusa* (V89) and clade III contains samples V9 and V29 of this species. The fourth large clade consists of *M. cactus*, *M. alta-septa*, *M. delicatula*, *M. hispida*, *M. stellata*, *M. florida* and unknown species 1 (sample V50) from Indonesia and some of the *M. hispida*, *M. peltiformis*, *M. stellata*, and *M. aequituberculata* samples from Magnetic Island. Clade V comprises four species [*M. cactus*, *M. capricornis*, *M. turtlensis* and unknown species 2 (sample V85)]. Clades VI and VII are small and consist of three (*M. capitata*, *M. danae*, and *M. verrucosa*) and four (*M. digitata* and *M. hoffmeisteri* from Indonesia and *M. mollis* and *M. spongodes* from Magnetic Island) species, respectively. Members of clade VI share two indels of 8 and 6 bp in length in the control region. These taxa also form a distinct clade based on *Pax-C* intron sequences (see below and Fig. 2B) together with an additional member, *M. hispida* M334.1, which clusters in clade IV in the mtDNA tree. Of the species for which we sampled

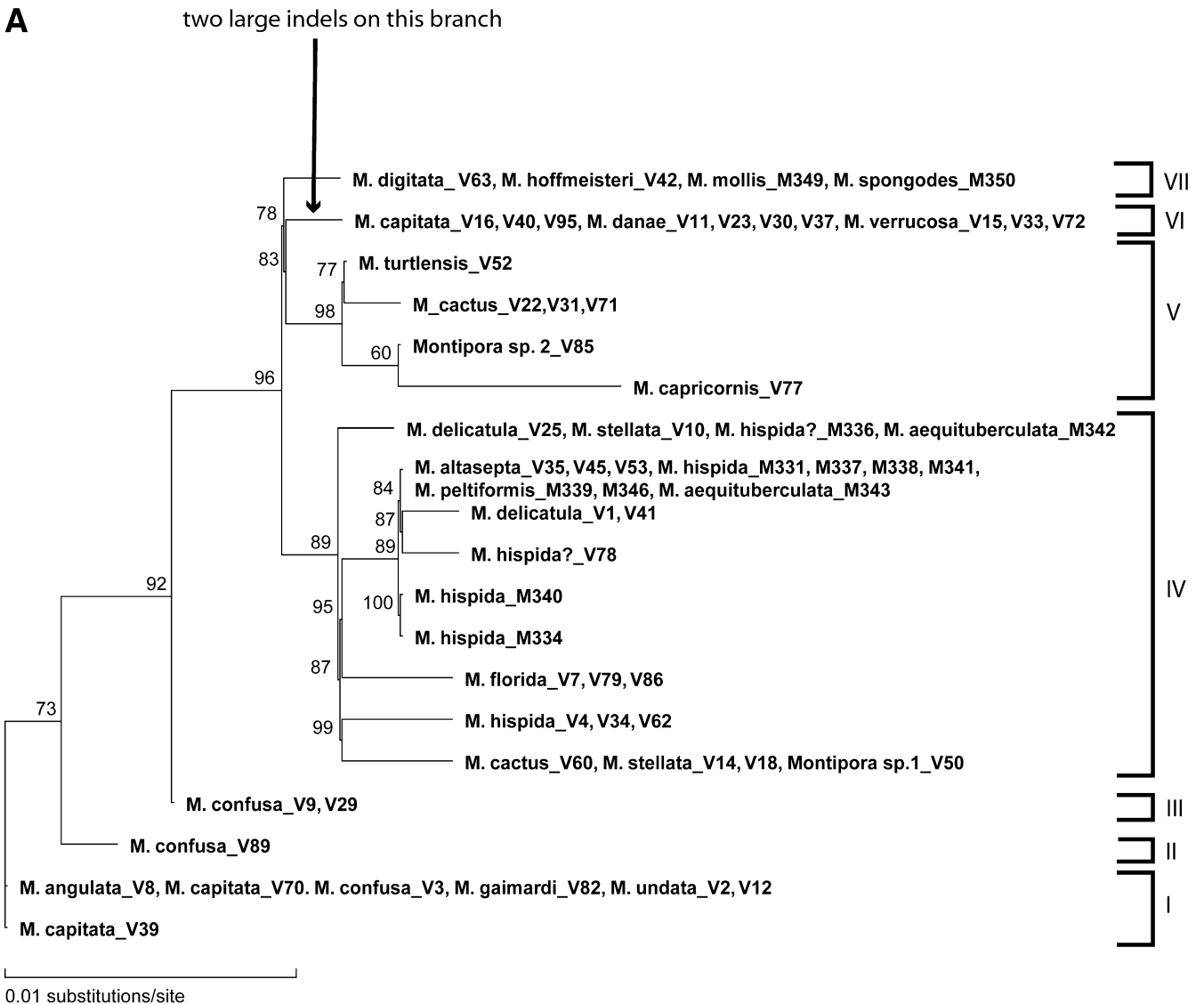
more than one colony, *M. aequituberculata*, *M. capitata*, *M. cactus*, *M. delicatula*, *M. stellata*, *M. confusa*, and *M. hispida* are not monophyletic based on mtDNA control

region sequences (although all colonies of each of the species *M. aequituberculata*, *M. delicatula*, *M. stellata*, and *M. hispida* cluster within the same main clade), whereas all colonies from *M. danae*, *M. verrucosa*, *M. altasepta*, *M. peltiformis*, *M. florida*, and *M. undata* have identical control region sequences. Enforcement of monophyly of individual species using Shimodaira–Hasegawa tests only resulted in a significantly worse tree in the case of *M. capitata* ( $P=0.044$ ). Despite this, the observation that some colonies have control region sequences that are identical to those of heterospecific colonies but differ from control region sequences of conspecific individuals is important. For example, the patristic distance between the haplotypes in *M. delicatula* V41 and V25 as calculated under statistical parsimony (with gaps excluded) in TCS version 1.13 (Clement et al. 2000) is 4, which is considerable given the overall low level of variation observed for the *Montipora* control region. In contrast, the *M. capitata* V16 sequence is identical to that of *M. danae* V11, V23, V30, V37 and *M. verrucosa* V15, V33, and V72.



**Fig. 1** Schematic overview of the gene order of a segment of the mitochondrial genome of *Montipora* spp. Numbers represent base positions within the *Montipora* putative control region. Asterisk denotes segment of control region of *Montipora* spp. that has an 88% similarity with a part of the *cox3* gene of *Acropora tenuis*

**Fig. 2** **A** Unrooted maximum likelihood tree of the *Montipora* mtDNA control region sequences. Values above the branches are bootstrap values (1,000 replicates). The arrow indicates the branch that is supported by two large indels. **B** Unrooted maximum likelihood tree of the *Montipora* Pax-C intron sequences. Values above the branches are bootstrap values (1,000 replicates). Designation of clades I–VII follows the mtDNA structure given in A. A dot and a number after the sample name indicates the clone that was sequenced (e.g. *M. cactus*\_V60.2 is *M. cactus* sample V60 clone 2)



**B**



**Fig. 2 (Contd.)**

*Pax-C* intron

The alignment of the *Pax-C* intron consists of 92 taxa (66 sequences from the V-series and 26 sequences from

the M-series) and 727 positions, of which 247 are variable and 120 are parsimony informative (gaps excluded; GenBank accession numbers AY313453–AY313544). Two sequences were not included in the alignment

**Table 4** Results of Shimodaira–Hasegawa test enforcing monophyly of individual species

| Species enforced to be monophyletic | Clade in which the species was placed | –lnL of constraint tree | Difference in –lnL between constraint and optimal tree | <i>P</i> value           |
|-------------------------------------|---------------------------------------|-------------------------|--|--------------------------|
| None (optimal tree)                 |                                       | 2845.3012               |  |                          |
| <i>M. capitata</i>                  | I, II, III                            | 2974.2099               | 128.9087   | 0.001 <sup>a</sup>       |
| <i>M. capitata</i>                  | VI                                    | 3068.7714               | 223.4702   | < 0.0000001 <sup>a</sup> |
| <i>M. cactus</i>                    | V                                     | 2959.8935               | 114.5922   | 0.037 <sup>a</sup>       |
| <i>M. cactus</i>                    | IV                                    | 2935.1438               | 89.8426  | 0.094                    |
| <i>M. delicatula</i>                | VI                                    | 2911.4422               | 66.1410  | 0.123                    |
| <i>M. delicatula</i>                | IV                                    | 2896.8989               | 51.5977  | 0.267                    |
| <i>M. confusa</i>                   | I, II, III                            | 2865.2458               | 19.9446  | 0.789                    |
| <i>M. hispida</i>                   | IV                                    | 3080.1618               | 234.8606   | < 0.0000001 <sup>a</sup> |
| <i>M. danae</i>                     | VI                                    | 2893.0593               | 47.7581  | 0.346                    |
| <i>M. verrucosa</i>                 | VI                                    | 2867.5010               | 22.1998  | 0.774                    |
| <i>M. peltiformis</i>               | IV                                    | 2903.5316               | 58.2304  | 0.255                    |
| <i>M. undata</i>                    | I, II, III                            | 2899.4588               | 54.1576  | 0.250                    |
| <i>M. altasepta</i>                 | IV                                    | 2916.6620               | 71.3607  | 0.085                    |
| <i>M. turtlensis</i>                | V                                     | 2912.0357               | 66.7344  | 0.163                    |
| <i>M. capricornis</i>               | IV                                    | 2863.0388               | 17.7376  | 0.876                    |
| <i>M. capricornis</i>               | V                                     | 2880.6929               | 35.3917  | 0.509                    |
| <i>Montipora</i> sp. 1              | IV                                    | 2876.5576               | 31.2563  | 0.578                    |

<sup>a</sup>Tree with enforced species monophyly significantly worse compared to optimal tree

because they are so divergent that large parts are unalignable. These are *M. delicatula* V25.1 (GenBank accession number AY313545) and *M. hoffmeisteri* V42.2 (GenBank accession number AY313546).

The HKY substitution model also shows the best fit to the *Pax-C* intron data, and a ML analysis was therefore performed in MolPhy using this model (Fig. 2B). As for the control region, the tree is unrooted due to the lack of suitable outgroup taxa. The *Pax-C* intron sequences contain several large gaps and to minimize information loss, an MP analysis with gaps treated as a fifth base was also performed. The resulting tree was similar to the ML tree presented in Fig. 2B. Moreover, a neighbour-joining-distance analysis in which gaps were deleted on a pairwise basis also yielded a tree topology similar to the ML tree.

Composition of the major clades in the *Pax-C* tree closely resembles that of the control region tree, albeit that the *Pax-C* intron tree shows more structure due to the higher levels of sequence variation in this region. A small number of taxa, however, cluster at different positions in the two phylogenies. For example, *M. delicatula* V41 clusters in clade IV in the mtDNA tree but is part of clade VI in the nuclear tree; *M. hispida* M334 groups in clade IV in the control region tree but in clades IV and VI based on the *Pax-C* data. Similarly, two *Pax-C* alleles of colony *M. capricornis* V77 fall in distinct main clades (IV and V). Of the species for which more than one *Pax-C* intron sequence was obtained, the following were not monophyletic: *M. altasepta*, *M. cactus*, *M. capitata*, *M. capricornis*, *M. confusa*, *M. danae*, *M. delicatula*, *M. hispida*, *M. peltiformis*, *Montipora* species 1, *M. turtlensis*, *M. undata*, and *M. verrucosa*. *Pax-C* intron sequences of *Montipora* species 2, *M. stellata*, *M. florida*, *M. mollis*, *M. angulata* and *M. mollis* are monophyletic. We tested whether trees in which individual species were enforced to form a

monophyletic group are significantly worse compared to the optimal tree (Table 4), and hence whether the support for non-monophyly of these species is compelling. Only trees in which *M. capitata*, *M. cactus*, and *M. hispida* were forced into a monophyletic cluster, respectively, were significantly worse compared to the optimal tree shown in Fig. 2B (Table 4). Hence, these three species are definitely not monophyletic based on the *Pax-C* intron. Note that the *Pax-C* intron sequence of *M. delicatula* V25.1 is largely unalignable with the other *Pax-C* sequences (including those of *M. delicatula* V41), indicating that this species is likely to be truly non-monophyletic as well.

## Discussion and conclusions

### Reticulate evolution in *Montipora*?

The mtDNA control region cannot resolve all species of *Montipora*. For example, the four *M. danae* colonies have identical control region sequences to the three *M. verrucosa* and three of the four *M. capitata* samples. This lack of resolution is probably a consequence of the low rate of evolution of mtDNA in Anthozoa (van Oppen et al. 1999; Shearer et al. 2002). Approximately half of the *Montipora* species studied share mtDNA haplotypes with other species, while some conspecific individuals have different haplotypes. We acknowledge that the sample sizes used are small for most species, but addition of more samples is unlikely to increase species monophyly, as some species have relatively divergent haplotypes (e.g. *M. capitata*) and several species share mtDNA haplotypes with other species, while some conspecific individuals have different haplotypes. Addition of new samples will affect haplotype frequencies and possibly increase the number of observed haplotypes,

but is unlikely to make currently non-monophyletic species, monophyletic. Although monophyly cannot be rejected for the majority of the species based on the more variable nuclear *Pax-C* intron, four species are likely to be truly poly- or paraphyletic. These are *M. capitata*, *M. cactus*, *M. hispida*, and *M. delicatula*. Non-monophyly of these species is supported by the mtDNA data (Fig. 2A). There are several explanations for these observations. Firstly, it is possible that lineage sorting has not yet reached completion (note that a slow substitution rate in mtDNA sequences does not imply that lineage sorting is slow as well). This is especially likely if species are of recent origin. As *Montipora* corals have delicate skeletons they do not readily form fossils and most species are therefore of unknown age. Nevertheless, the genus as a whole is old, known to occur as far back as the Oligocene of the Tethys (Frost 1977) and possibly the Eocene of the central Indo-Pacific (Gregory and Trench 1916) (an interval of at least 30 million years). Only two species of pre-Pleistocene *Montipora* have been identified with any degree of certainty. One of these species is *M. aequituberculata* from a Pliocene (2–3 million years old) outcrop of Papua New Guinea (Veron and Kelley 1988), and this species shares mtDNA haplotypes with a range of congeners (Fig. 2A). The question is whether 2–3 million years is likely to be sufficient for alleles to sort into the different species in the absence of interspecific hybridisation, which is difficult to assess without knowing generation times or effective population sizes. In contrast to massive corals, such as *Porites*, *Montipora* corals are believed to have relatively short generation times as these are fast-growing corals that reach reproductive maturity at a few years of age. *Montipora* colonies are probably not very long-lived and a slow-down of lineage sorting due to the overlap of generations is therefore unlikely. Species for which genetic monophyly cannot be rejected are intermingled with truly non-monophyletic species. This indicates that the observed patterns of non-monophyly are unlikely to be due to incomplete lineage sorting, although it cannot be unequivocally excluded at this stage. A second possible explanation for the pattern of non-monophyly is morphological convergence, or the existence of cryptic species that are not taxonomically recognizable. The third interpretation is that unknown *Montipora* species have exchanged genes through interspecific hybridisation, leading to the formation of reticulate patterns of evolution. These patterns will not be morphologically recognizable in any single region, but they are recognizable over the large geographic ranges most species have (Veron 1995). They would, however, create non-monophyly. Like *Acropora*, *Montipora* species are known to participate in the annual mass-spawning event on the GBR (Harrison et al. 1984; Babcock et al. 1986) and some simultaneous spawning has also been recorded in Hawaii (Heyward 1986), Japan (Hayashibara et al. 1993), and Indonesia (A. Baird, personal communication). Moreover, two morphologically distinct species have been shown to interbreed

successfully in vitro (Willis et al. 1997). Although these data on spawning times and cross-fertility in *Montipora* are limited, they suggest that opportunities for introgressive hybridisation may exist in nature between at least a small number of species. The genetic non-monophyly for the four species *M. capitata*, *M. cactus*, *M. hispida*, and *M. delicatula* is compatible with the occurrence of occasional hybridisation, as are the topological differences between the mitochondrial and nuclear trees.

Although hybridisation may occur between a small number of *Montipora* species, for the majority of species examined here monophyly cannot be rejected, suggesting these are good biological species that do not hybridise. Interspecific breeding is probably limited by mechanisms such as gamete–gamete recognition and chemical attraction between eggs and sperm of the same species. Sperm-attractant molecules have been isolated from eggs of *Montipora* species and although *M. tortuosa* and *M. digitata* were attracted to the same compound, the morphologically more distinct *M. crassituberculata* was not (Coll et al. 1994). Several other scleractinians, including some that participate in mass spawning, also appear to be good biological species. For example, the species pair *M. digitata* and *M. tortuosa* are distinguished by fixed allelic differences in sympatry (Stobart and Benzie 1994; Stobart 2000). *Acropora* (*Isopora*) *palifera* and *A. cuneata*, both of Pliocene origin (Wallace 1999), also show fixed allozyme differences in sympatry on Magnetic Island in the central GBR (Ayre et al. 1991). Five *Porites* spp. and two *Goniopora* species were also found to be genetically distinct based on 13 allozyme loci (Garthwaite et al. 1994).

#### Morphology and genetic diversity in *Montipora*

A cladistic analysis of morphological characters is absent for *Montipora* spp. Nevertheless, species have been divided into growth groups based on growth form and skeletal characters (Veron 2000). A comparison of these growth groups and the genetic data presented here suggests that morphological and molecular relationships are largely incongruent in *Montipora*, although some agreement exists. For example, *M. capitata*, *M. danae*, and *M. verrucosa* belong to the same growth group and have similar morphological structures (Veron 2000). In both molecular trees (Fig. 2A, B) these species form a distinctive group supported by two (8 and 6 bp in length) shared indels in the control region (clade VI), with the exception of samples *M. capitata* V39, *M. capitata* V70 and the additional presence of a sequence of *M. hispida* M334.1 in the *Pax-C* clade VI. *M. confusa* has a strong resemblance to *M. undata* and the two species group together in both trees. *M. hispida* is a taxonomic wildcard (J.E.N. Veron, personal observation) and has an extreme range of growth form and continuous geographic variation, which is consistent with it clearly being genetically polyphyletic. In contrast,

*M. hispida* and *M. cactus* have similar micro-skeletal detail but are not closely related genetically. There are several possible reasons for the disparity between genetic and morphological affinities. Firstly, phenotypic plasticity is common in scleractinian corals, and some *Montipora* species show continuous rather than discrete variation in skeletal characters. Secondly, it is possible that the morphological attributes used to define growth groups show high levels of homoplasy. Thirdly, interspecific hybridisation can decouple genetics from morphology. Our data are consistent with a small number of species occasionally exchanging genes with congeneric species.

Pairwise genetic distances in the *Pax-C* intron and the mtDNA control region are a factor 2–4 smaller in the genus *Montipora* compared to *Acropora* (Table 3). Diversity in the *Montipora Pax-C* intron is an underestimate of the true diversity, as two largely unalignable alleles were not taken into account in this estimate. However, such alleles are rare. The difference in diversity between the two genera is unlikely to be a consequence of geological age, which is similar for the two genera. The oldest known *Acropora* is from the Eocene (~40 million years old) of the Caribbean, Pacific, and Tethys. *Montipora* may well have a similar age although the earliest unequivocal remains are Oligocene (~30 million years old) of the Tethys (Veron 1995). Difference in genetic diversity between these two genera may be due to differences in mutation rates or generation times, but further studies are required to address this question.

## Conclusions

Returning to the questions set out at the start of this study, we can conclude that in the scleractinian genus *Montipora* little agreement exists between morphological species and systematic units determined from both nuclear and mitochondrial DNA. The non-monophyly of some species as well as the inconsistencies between nuclear and mitochondrial phylogenies suggests that gene exchange has taken place between a small number of species through introgressive hybridisation and that this has led to the formation of reticulate patterns.

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